EVALUATION AND COMPARISON OF COMPOSITION AND BIOLOGICAL ACTIVITIES OF PEPTIDES OBTAINED FROM RAINBOW TROUT (*Oncorhynchus mykiss*) AND CHLORELLA (C*hlorella sorokiniana*)

By

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ABSTRACT

This study compared protein hydrolysates generated from Rainbow Trout (*Oncorhynchus mykiss*) by-products and *Chlorella* (*Chlorella sorokiniana*), for their functional properties as bioactive peptides. The Trout hydrolysate was derived via a fermentative hydrolysis method, while *Chlorella* hydrolysate was obtained by sequential microwave treatment and enzymatic hydrolysis. Both hydrolysates were fractionated using Ultrafiltration membranes. Lyophilized crude hydrolysate and different molecular weight fractions were analyzed for antioxidant and Angiotensin converting enzyme (ACE) inhibitory capacities. Despite similar protein content (30% dw) in *Chlorella* biomass, the hydrolysate's soluble protein was 4.96 mg/mL, nearly 90% lower than the soluble protein content of fish hydrolysate. Nevertheless, *Chlorella* hydrolyzate fractions showed significantly higher antioxidant capacities compared to fish hydrolysates, while no difference was observed for ACE inhibition activity. Protein extraction from *Chlorella* could be enhanced and optimized to increase yield. Supplementation of trout hydrolysate with *Chlorella* hydrolysates was identified as potentially providing a unique high-value bioactive peptide blend for combined activities.

Keywords: *Bioactive Peptides, Trout by-products, Chlorella peptides, antioxidant capacity, protein hydrolysis*

LIST OF ABBREVIATIONS AND SYMBOLS USED

α	Alpha
β	Beta
°C	Degree Celsius
%	Percentage
<	Less than
>	Greater than
~	Approximate
µL	Micro litre
AOAC	Association of Official Analytical Chemists
Cfu	Colony forming unit
g	Gram
h	Hour(s)
HCl	Hydrochloric acid
HPLC	High Pressure Liquid Chromatography
L	Litre
M	Molar
Min	Minute(s)
Mg	Milligram(s)
mL	Milliliter(s)
mM	Millimolar
NaOH	Sodium hydroxide
w/v	Weight per volume

dw	Dry weight basis
ACE	Angiotensin converting enzyme
DPP-IV	Dipepidyl-peptidase IV
DPPH	2-2-diphenyl-2-picrylhydrazyl
ABTS	2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate)
ORAC	Oxygen radical absorbance capacity
UVC	Ultraviolet C
UF	Ultrafiltration
NF	Nanofiltration
MWCO	Molecular weight cut-off
LC-MS/MS	Liquid chromatography- tandem mass spectrometry
UPLC-MS/MS	Ultrahigh performance liquid chromatography tandem mass spectrometry
Q-TOF	Quadrupole time-of-flight
ESI	Electrospray ionization
ROS	Reactive oxygen species
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
BHA	Butylated hydroxy anisole
BHT	Butylated hydroxytoluene
PG	Propyl gallate
TBHQ	Tert-butyl hydroquinone
LAB	Lactic acid Bacteria

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CHAPTER 1: INTRODUCTION

1.1. Thesis background

Bioactive compounds are those food components that influence the body as whole or specific tissues or cells to promote good health (1). Among the bioactive compounds, bioactive peptides have piqued the interest of industry, academia, and the general public with their ability to influence major physiological functions such as antioxidative, antithrombotic, antihypertensive, and immunomodulatory activities (2). Bioactive peptides are commonly described as protein fragments ranging from 2 to 20 amino acid units, with a positive impact on human physiological functions or conditions, and may ultimately influence health beyond their nutritional capabilities (2). Thus, over the last few years, there has been an increased scientific interest in finding unique bioactive peptides that can reduce or prevent the risk of chronic diseases and provide immune protection. As a result, extensive research has been dedicated to the processing and generating bioactive peptides from under-utilized protein-rich by-products of the food industries (3–5). Using underutilized protein-rich by-products for bioactive peptide production is highly beneficial since it does not compete with the food supply and provides solutions for waste management problems (6,7). Marine by-products generated from fish, crustaceans, and algae have gained the attention of researchers for the production and utilization of proteins and peptides (8,9). Among the marine sources, fish by-products are a significant source of protein hydrolysates and bioactive peptides (10). Industrial processing of fish for human consumption yields ~60% by-products as processing leftovers, including trimmings, fins, frames, heads, skin, and viscera (11). Even though the average protein content of fish ranges between 9 - 24% on a wet weight basis (10), the literature shows that fish by-product

protein contents tend to be lower (6 – 18%) due to the removal of muscle tissue during processing (12). Fish by-products are a sustainable resource for generation of bioactive peptides (13) however supply is limited by seasonal variations (14–16) and fish harvest fluctuations (17). These reasons can affect the supply of fish by-products leading to a need to identify readily available and complementary supplementary resources. A potential supplementary protein source, microalgae have been shown to provide a range of bioactive products such as carotenoids (18), phycobilins (19,20), fats (21), polysaccharides (22,23), vitamins (24,25), sterols, and peptides (26,27). The current production of microalgae is mainly focused on the biofuel markets, niche nutritional supplements, and the aquaculture feed industry (28). Most of these applications do not maximally utilize the valuable protein fraction of the microalgae. An ideal biorefinery for microalgae utilization would focus on biomass extraction using a step-by-step approach to extract all the different fractions to convert into marketable products (29). Microalgae biomass composition ranges by species, with some strains containing up to 60% carbohydrates (30,31), where other species can contain up to 70% lipids (32,33) or ~60% proteins (34). Among the microalgae, *Chlorella* spp are extensively studied for biofuel production (34).

Additionally, *Chlorella* spp is also used for protein extraction because of its high protein content (51% - 58% dw) (34) to be used in pharmaceutical, food, cosmeceutical, and bioenergy applications. Therefore, *Chlorella* spp may provide a suitable sustainable source of bioactive peptide production to supplement fish by-products and can be cultivated through a range of methods at an industrial scale (35). *Chlorella* protein composition and quality might also be expected to be distinct from that of fish by-product proteins though the peptide content of the latter is largely unquantified. However, bioactive

peptides from microalgae (*Chlorella sorokiniana*) peptides may exhibit functionality that is either comparable or higher in functionality to peptides generated from fish (Rainbow trout (*Oncorhynchus mykiss*)) by-products. Therefore, specific strains could be considered as a potential candidate not only to overcome the supply limitations of marine biomass but to biologically produce marine - like peptides which have the capacity to serve the need for a range of functional ingredients in food.

Utilizing marine bioprocessing waste to produce food-grade protein hydrolysates/ bioactive peptides is a promising approach in Atlantic Canada due to plentiful marine resource availability. Therefore, it is essential to understand the significant differences of derived peptides generated from different sources. More specifically, the production of microalgae and fish by-product derived, bioactive-rich supplements or nutraceuticals with functionalities of antioxidant and antihypertensive properties can contribute to ongoing efforts to reduce the global concerns of health issues.

1.2. Objectives

1. To compare and characterize peptide yield resulting from the hydrolysis of proteins from Chlorella (Chlorella sorokiniana) and Rainbow trout (Oncorhynchus mykiss) by-products.
2. To evaluate and compare the composition of Chlorella (*Chlorella sorokiniana*) and Rainbow trout (*Oncorhynchus mykiss*) hydrolysates.
3. To evaluate and identify specific bioactivities of Chlorella (*Chlorella sorokiniana*) and Rainbow trout (*Oncorhynchus mykiss*) protein hydrolysates and fractionated peptide samples.

1.3. Thesis organization

The thesis is organized into five chapters. Chapter 1 introduces the project and outlines the project objectives. Chapter 2 reviews relevant literature in the field of study, including information on past studies on fish by-products, micro algae, bioactive peptides, and their antioxidant and antihypertensive activity. Chapter 3 provides information on the experimental details of the generation of bioactive peptides, the characterization methods of the bioprocessing approach, and the bioactivities of obtained hydrolysate and peptide fractions. Chapter 4 presents the study outcomes with a comparison to previously published reports. Chapter 5 presents the conclusions drawn from the current work and future recommendations.

CHAPTER 2: LITERATURE REVIEW

2.1. Fish by-products

Fish by-products refer to tissues/parts that remain following processing for fillets and include heads, frames, viscera, and skin, which comprise around 60% of the total fish weight (11). Currently, fish by-products are used primarily to produce fish meal and fish oil (36), and fish skin is converted to gelatin or fish leather (37,38). Fish by-products are also used to make fertilizer and other products such as silage, biodiesel/ biogas, and natural pigments (39,40). However, the primary uses of fish meals and oils include aquaculture feed ingredients for fish and shrimp and livestock and poultry feed ingredients (36). Although they are used in fish oil production, the edible and protein-rich components in these by-products remain underutilized in low-value commodities. Moreover, significant quantities of the fish processing wastes are disposed of at sea from factory processing vessels (17), which significantly impacts the environment. Due to the high amount of organic matter in these wastes, there has been a significant impact on the native biodiversity in marine ecosystems (40). Even though fish and fish by-products vary significantly in their composition and yield, which also depends on the season and geography (41), these by-products remain inexpensive and a sustainable reservoir of edible proteins that can be used to produce bioactive peptides. The added value from utilizing these wastes can reduce food processing costs, with the additional advantage of generating innovative functional bioproducts.

The processing of fish has a rich history that dates to medieval times. Traditional preservation techniques include drying, salting, pickling, or fermentation (42). Based on

the nature of the biologically induced changes occurring in the products, the techniques can be summarized into two major categories; enzyme hydrolyzed and bacterially fermented (42). Several methods such as acid and alkaline hydrolysis, autolysis, microbial fermentation, and enzymatic hydrolysis have been studied to generate peptides from fish by-products in the past (43,44).

Among the different fish species that can be used to produce protein hydrolysate, Rainbow trout (*Oncorhynchus mykiss*) holds the second position after salmon in Canada, being one of the popular farmed fish species in the industry, with an annual average of around 8,750 tons of trout per year produced in the last five years (2015-2019) in Canada, and a yearly average of 412 tons over the previous five years in Nova Scotia (2015-2019) (45). Therefore, utilizing trout as a hydrolysate/ peptide generation source has attracted significant interest due to reported bioactivities as described by studies listed in Table 1.

Table 1: Reported bioactivities from different by-products of Rainbow Trout (*Oncorhynchus mykiss*)

By-product	Process	Reported Activity	Reference
Collagen peptide from Trout skin	-	Decrease in lipid absorption and metabolism and lower levels of plasma total lipids and triglycerides in rats	(46)

Frames	Enzymatic Hydrolysis (Alcalase®)	Antioxidative activity (2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging)	(47)
Skins	Enzymatic Hydrolysis (Alcalase®)	Antioxidative activity (DPPH radical scavenging, ABTS radical scavenging, Reducing power)	(48)
Roe	Enzymatic Hydrolysis (Alcalase® and Pepsin)	Antioxidative activity (DPPH radical scavenging, Ferric reducing antioxidant power)	(48)
Frames	Microwave treatment and enzymatic hydrolysis (Alcalase®)	Antioxidative (DPPH radical scavenging, Ferric ion reducing antioxidant capacity)	(49)

Frames	Microwave Pretreatment and Assisted Enzymatic Process (Alcalase®)	Antioxidative (2,2′-Azino-Bis(3-Ethylbenzthiazoline-6-Sulphonic Acid) Free Radical Scavenging Assay, Reducing Power, Metal Chelating Activity)	(50)
Muscle	Enzymatic Hydrolysis (Alcalase, α -chymotrypsin, Neutrase, papain, pepsin, and trypsin)	Angiotensin I converting enzyme (ACE) inhibitory activity	(51)
Frames	Microwave treatment and enzymatic hydrolysis (Alcalase®)	ACE inhibitory activity and Dipeptidyl-peptidase IV (DPP-IV) inhibition	(52)

Skin	Enzymatic Hydrolysis (Alcalase® and Flavourzyme®)	Antioxidative (DPPH radical scavenging) and Anticancer activity	(53)
By-product mix	Autolysis	Antioxidative (Free radical scavenging activity and the metal chelating ability)	(54)

As shown in Table 1, trout protein hydrolysates have been reported to demonstrate antioxidant, anticancer, and antihypertensive activities. These studies utilized different parts of by-products to generate the protein hydrolysates and the byproducts together as a mix. In these studies, angiotensin-converting enzyme (ACE) and dipeptidyl-peptidase IV (DPP-IV) inhibitory activity, antioxidant activities (free radical scavenging activity and the metal-chelating antioxidant activities), and notable anticancer effects were reported from hydrolysates generated using trout by-products. In addition, in another study reported that trout hydrolysates generated from viscera demonstrated antibacterial activity against *Flavobacterium psychrophilum* and *Renibacterium salmoninarum* prolonging the lag phase of bacterial growth (55).

2.2. Microalgae

The markets for microalgae have been mainly focused on biofuel production (28) and shifted the focus towards higher-value products from biomass such as the production of

single-cell protein, niche nutritional supplements, alginates extraction, and aquaculture feed. However, the biorefinery concept, i.e., production of bioproducts including fuels, energy, materials, chemicals, and nutraceuticals from biomass by integrating the downstream processing techniques as cost-effective and environmentally friendly manner microalgae is emerging. The primary reason is that biofuels from microalgae are still challenged to achieve economic feasibility due to overwhelming capital investments and operations (29). Hence, a biorefinery concept supports high-value co-product production through the sequential extraction of various products to improve the economics of a microalgae biorefinery. However, high protein microalgae are an untapped sustainable natural resource for innovative natural functional ingredients (28) with 40 - 60% (dw) protein content, particularly for bioactive peptides (56). The market for microalgae biomass is expanding with the advent of new technologies in cultivating, handling, and utilizing microalgae biomass (57).

Among different microalgae species, it is reported that the *Arthrospira* sp. contains the highest recorded protein content of ~60% (dw) (58). *Chlorella* sp., *Scenedesmus obliquus*, *Spirulina* sp., and *Athrospira* sp. are some of the species selected for large-scale production (34). *Chlorella* species are widely researched for biofuel and bioactive peptide production due to their versatile growing ability and relatively high protein and lipid contents (59). Peptides from *Chlorella sp.* have been reported to exhibit multiple bioactivities, as reported in Table 2.

Table 2: Peptides derived from microalgae *Chlorella* sp.

Species	Reported bioactivity	Hydrolytic method	Bioactive amino acids or peptide sequences	Reference
Chlorella vulgaris	Anti-oxidative (DPPH radicals scavenging activity, Hydroxyl radicals scavenging, Superoxide radicals scavenging, Oxygen radical absorbance capacity (ORAC))	Enzymatic – Pepsin	Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Glu-Phe	(60)
Chlorella vulgaris	ACE inhibitory, Anti-oxidative (Superoxide radical quenching)	Enzymatic – Pepsin	Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Glu-Phe	(61)
Chlorella vulgaris	Anti-proliferation	Enzymatic – Pepsin	Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Glu-Phe	(62)
Chlorella vulgaris	ACE-I inhibitory	Enzymatic – Pepsin	Ile-Val-Val-Glu	(63)

Chlorella ellipsiodea	Antioxidant activities (DPPH radical scavenging, Hydroxyl radical scavenging, Peroxyl radical scavenging)	Enzymatic Pepsin	Leu-Asn-Gly-Asp-Val-Trp	(64)
Chlorella pyrenoidosa	Antioxidant activities (UVC-Induced Cytotoxicity)	-	Peptide mixture	(65)
Chlorella sorokiniana	ACE-inhibitory	Protease N	Trp-Val, Val-Trp, Ile-Trp, and Leu-Trp,	(66)

Extracts from *Chlorella* have demonstrated different antioxidant and antihypertensive properties in their protein hydrolysates and peptides. It can be observed that different studies have reported different peptide sequences. A recent study on the prediction of bioactive peptides yielded after protease cleavage using BIOPEP-UWM Tool from *Chlorella sorokiniana* has suggested that proteins of *C. sorokiniana* have the highest number of dipeptidyl peptidase-IV (DPP IV) inhibitors, with a high occurrence of other bioactive peptides such as angiotensin-I converting enzyme (ACE) inhibitor, glucose uptake stimulant, antioxidant, anti-amnestic and antithrombotic peptides (67).

Algal protein biomass residual from oil extraction is often used only as a protein source in animal feed (59). Sankaran et al. (57) introduced the term spent microalgal biomass

(SMAB) and reviewed the ways to utilize the SMAB extensively. To better utilize *Chlorella* biomass, proteins need to be extracted and hydrolyzed. It can be broken down mainly to microalgae cell wall disruption to gain access to the intracellular proteins, hydrolysis of proteins, and extraction or isolating peptides obtained from hydrolysis (59) (Figure 1).

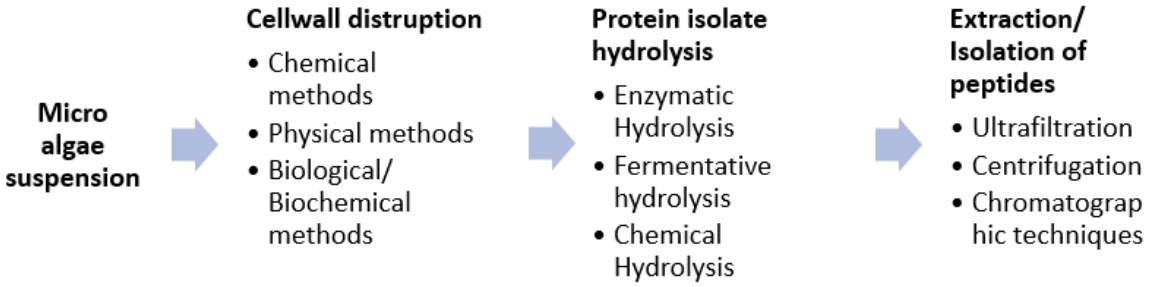

Figure 1: Methods for production of bioactive peptides from microalgae

2.3. Amino acid composition of hydrolysates

Protein hydrolysates obtained after hydrolysis of proteins are composed of free amino acids and short-chain peptides exhibiting many advantages as nutraceuticals or functional foods because of amino acid sequences in peptide chains. The amino acid sequence of proteins has significant roles in various physiological and biological activities within cells and tissue, impacting metabolic function. Therefore, the composition of available amino acids is significant in the synthesis of a wide variety of proteins with different amino acid sequences, which perform important functions such as carriers of oxygen, vitamins, CO_2, enzymes, and structural proteins. This variation in the amino acid composition of available proteins influences protein hydrolysis generated peptides and the generations of different short-chain peptides. It depends on several factors, such as enzyme source and hydrolysis

conditions (68,69). Therefore, different sources of proteins generate different short-chain peptides when hydrolyzed due to differences in inherent amino acid sequences in the parent protein. Fish and microalgae have both been identified as well-balanced protein sources due to the availability of all essential amino acids in their amnio acid profile (34,70). Among all the amino acids, aspartic acid and glutamic acid comprised a higher proportion in most of the reported fish protein hydrolysates (68,71–73). Fish head, skin, and visceral hydrolysates were reported to contain all the essential and non-essential amino acids (71,74–76), whereas aromatic amino acids were not reported in fish frame protein hydrolysates (77). Becker (34) listed the amino acid composition of several micro algae species including *Chlorella*, and compared it with egg and soybean. It is observed that the amino acid pattern of almost all algae compares favorably with that of the reference food proteins. *Chlorella* hydrolysates have also been reported to have higher arginine, glutamic acid, glycine, and cysteine contents in their peptide fractions (78,79). Hence, the protein hydrolysates produced from different parts of fish and *Chlorella* can generate diverse peptides due to the excellent amino acid composition in raw materials.

2.4. Enhanced selectivity of protein hydrolysates using ultrafiltration

Several methods are widely used to obtain hydrolysate enriched in bioactive peptides with a high yield (or to isolate potent bioactive peptides), including size-exclusion chromatography, ion-exchange chromatography, liquid chromatography, and ultrafiltration (UF) using different kinds of UF membranes (with a range of MWCO; 100, 20, 10, 5, 3, and 1 kDa molecular weight cut off (MWCO) membranes) (80). These methods can separate peptides in the protein hydrolysates based on physicochemical parameters such as molecular size, hydrophobicity, and charge.

Pressure-driven membrane separations such as UF have been used with protein hydrolysis to increase the specific activities of the protein hydrolysates. UF techniques offer advantages of lower cost and ease to scale up for commercial production. This system has the main advantage that the molecular weight distribution of the desired hydrolysates can be controlled by adopting a suitable UF membrane (81).

Several studies have used membrane processing to obtain biologically active fractions. For example, the application of the multilayer membrane system showed the potential to recover high ACE-inhibitory activity from tilapia by-product protein hydrolysate (82). In another study, UF at 100, 30, and 10 kDa have been used to effectively recover protein with bioactive properties from cuttlefish wastewater (83). The antioxidant activities of tuna dark muscle by-product hydrolysate and its peptide fractions were evaluated by UF and nanofiltration (NF) membrane processes (84). Similarly, *Chlorella sorokiniana* protein isolates were enzymatically hydrolyzed using pepsin, bromelain, and thermolysin, and fractionation of protein hydrolysates was carried out by UF membrane with MWCOs of 5 and 10 kDa before determining bioactivities (85). *Chlorella ellipsoidea* protein hydrolysate was fractionated into three ranges of molecular weight (below 5 kDa, 5–10 kDa, and above 10 kDa) using UF membranes to determine ACE inhibitory activity (86). These studies successfully isolated bioactive peptide fractions utilizing UF membranes to fractionate the protein hydrolysate of interest (87–90).

2.5. Qualitative and quantitative analysis of peptides

Following consecutive purification processes, antioxidant peptides are usually sequenced by one of a number of methods, such as liquid chromatography-tandem mass

spectrometry (LC-MS/MS) (91) or ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (92). LC-MS/MS techniques enable successful identification of antioxidant peptides using a quadrupole time-of-flight tandem mass spectrometer (Q-TOF) equipped with an electrospray ionization (ESI) source, which runs in the positive ion mode (93). Alternatively, a Matrix-assisted laser desorption/ionization-time of flight spectrometer (MALDI-TOF-MS/MS) can be used to identify antioxidant peptides, which have been purified from protein hydrolysates (94).

Bioinformatics is an innovative, supportive and systematic strategy developed to overcome cost-intensive and time-consuming conventional bioactive peptide investigation methods. This approach uses several databases, such as NCBI, BIOPEP-UWM, and BLAST (databases), containing different information about the amino acid sequence of a wide range of various parent proteins and peptides, frequency of bioactive peptides occurrence, and specific enzymes to be used for releasing peptides with predicted activities and properties. Furthermore, these databases can also anticipate the physicochemical, functional, and sensory properties of the predicted peptides.

The protein's amino acid composition and sequence greatly determine the presence of antioxidant peptides with required bioactivities (67). The protein's amino acid sequence can be obtained from databases, such as UniProtKB and NCBI, if the protein source is studied and reported that the hydrolysate from this protein exhibited particular bioactivity. Darewicz, Borawska, and Pliszka (95) used the UniProtKB database to retrieve the amino acid sequence of Carp protein to predict potential antioxidant peptides. Alternatively, proteomic techniques can analyze and characterize amino acid sequences of unknown proteins from new source materials (96). In recent years, a mass spectrometer (MS) based

proteomics technique has been successfully used to determine and identify various proteins from food materials (67,97,98). The sequence obtained from MS techniques can be further observed using BLAST (basic local alignment search tool) analysis. BLAST analysis generates data of "identities", "positives," and "gaps" of two or more aligned proteins (98). It can be used to examine the homology of the identified protein by aligning with similar proteins of other materials obtained from the NCBI database.

Once the protein is identified and characterized, sequences are subjected to in silico analysis in the BIOPEP-UWM database, where simulation of enzymatic hydrolysis is performed to predict bioactive peptides theoretically released from the intact protein sequence, as well as potential enzymes possibly used to release the peptides. According to the study by Tejano et al. (67), the application of the BIOPEP-UWM database revealed that a high number of DPP IV inhibitors could be obtained from *Chlorella sorokiniana* proteins identified by LC-ESI-MS/MS. The study also used in silico analysis to simulate enzymatic hydrolysis and revealed that pepsin, bromelain, and papain peptidases released many antioxidant peptides.

2.6. Bioactive peptides

Bioactive peptides refer to various amino acid sequence fragments, normally comprising 2–20 amino acids (2), traditionally produced from food proteins and non-food protein sources (wastes and underutilized sources) that can undergo hydrolysis. Bioactive peptides are active fragments, but they remain inactive as long as they stay locked in the parent protein (99). These are generated directly by enzymatic hydrolysis with commercial enzymatic preparations or indirectly during processing steps such as fermentation and

ripening (100,101). Bioactive peptides also result naturally from digestion and degradation by various proteases, including gastric and pancreatic proteases, brush border proteases, and cytosolic and plasma proteases. Intestinal cells must absorb bioactive peptides from the gastrointestinal lumen and release the absorbed peptides into circulating blood for the peptides to arrive at organs or biological systems. Then properties such as charge, size, and secondary structure enable these peptides to interact with receptors or other biomolecules such as signals, hormones, and enzymes to modulate their physiological functions (101,102).

Recent studies have revealed that peptide molecular weight and amino acid sequences affect the bioactivity of peptides. Furthermore hydrophobicity, charge, and functional groups present in the amnio acids impact their bioactivity. For example, Mendis et al., (103) indicated that hydrophobic amino acids present in the structure of the peptide affect the antioxidant activity of a peptide in inhibiting lipid peroxidation; they also reported that hydrophobicity leads to high interactions between the peptide and the fatty acids, concluding in protection against oxidation. Rajapakse et al., (104) reported that amino acids with aromatic residues improve the radical-scavenging properties of the peptides because they can donate protons to electron-deficient radicals. However, Mundi and Aluko, (105) reported that higher DPPH inhibitory activity for peptide fractions with higher contents of hydrophobic aliphatic (Val, Iso, and Leu) and hydrophobic aromatic (Phe and Tyr) amino acid residues. In addition, the overall charge of a peptide affects its ability to form hydrogen bonds with water and affects solubility and hydrophobicity. It is noted that peptides with polar and ionizable groups were more soluble (106).

The health-related benefits of bioactive peptides are attributed to their physiological activities exerted in vitro and in vivo; these include lowering blood pressure by inhibiting the angiotensin-converting enzyme (ACE); oxidative stress reduction by neutralizing or scavenging free radicals; enhancing mineral absorption by functioning as carriers for different minerals (e.g. calcium and iron) and reducing the risk of obesity and type 2 diabetes by inhibiting enzymes associated with the disease development (2,107–109). Therefore, when exploited, bioactive peptides could be beneficial for the treatment and management of numerous lifestyle-related diseases.

2.6.1. Antioxidant activity

Reactive oxygen species (ROS), such as hydroxyl radicals (·OH), superoxide anion radicals ($O_2 \cdot^-$), nitroxide radicals (NO·), peroxyl radicals ($ROO \cdot^-$), and hydrogen peroxide (H_2O_2), are a series of chemically reactive products of stepwise reduction of oxygen (110). Oxidative stress refers to a state with an imbalance between ROS and antioxidant systems, which causes damage to biomolecules. (111). ROS are normally generated as by-products of oxygen metabolism; despite this, environmental stressors (i.e., UV, ionizing radiations, pollutants, and heavy metals) and xenobiotics (i.e., antiblastic drugs) contribute to increasing ROS production significantly, therefore causing the imbalance that leads to cell and tissue damage (oxidative stress). Therefore, it is considered to be an important factor contributing to cancer, diabetes, neurological malfunction, and weakening of the immune system, which is manifested by lipid peroxidation, free radical formation, protein oxidation, nitrotyroine, or DNA/RNA oxidation (112). Antioxidant peptides can defend the human body against damages created by ROS as a result of complex interactions between their ability to inactivate ROS, scavenge free radicals

(113,114), chelate pro-oxidative transition metals (115), reduce hydroperoxides (116), and enzymatically eliminate specific oxidative agents (117).

A range of natural antioxidant compounds (phenolic compounds, tocopherol, and ascorbic acid) have been identified and are utilized in the food industry. Specific bioactive peptides from natural proteins are also considered as an alternative to synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butyl hydroquinone (TBHQ). Increasing evidence is being presented relating to the antioxidant activities of peptides with their amino acid sequences (118). Wu et al., (111) reviewed the amino acid composition, molecular weight, and degree of hydrolysis for the antioxidant properties of the peptides and their relation to anti oxidative capacity. They suggested that in addition to low molecular weight and a high degree of hydrolysis, the presence of oxidizable fragments (double bonds, indole groups, hydroxyphenyl groups, and sulfhydryl groups) in amino acids increases the ability of peptides to act as an antioxidant. However, oxidation of proteins typically results in the modification of amino acid R-groups, polymerization, and fragmentation reactions of proteins are also possible in the presence of oxygen (119). Cleavage of proteins into peptides will improve the exposure of the amino acid R-groups; therefore, the antioxidative property of the peptides is relatively higher than that of proteins.

Comprehensive reviews have been published on the production of antioxidant peptides from different marine resources such as algae, oysters, mussels, squid, and different species of fish (120–122). Most studies evaluated the antioxidant activity of the hydrolysates/peptides *in vitro* using different methods such as DPPH (1,1-Diphenyl-2-picrylhydrazyl) scavenging activity, reducing power, ABTS (2,2'-azino-bis(3-

ethylbenzothiazoline-6-sulfonic acid)) scavenging activity, Fe^{2+} chelating activity, β-carotene bleaching preventing activity or linoleic acid autoxidation inhibition activity (70). Some studies also investigated the ability of the hydrolysates/peptides to inhibit lipid oxidation in real food systems such as fish oil in water emulsions (123–125) or fish oil microcapsules (126,127) found out that fish protein hydrolysates are capable of physical and oxidative stabilization of those emulsions.

2.6.2. Antihypertensive activity

Angiotensin-converting enzyme (ACE) is one of the primary enzymes responsible for blood pressure regulation through a mechanism called the renin-angiotensin system as well as for electrolyte homeostasis (128). ACE is a nonspecific dipeptidyl carboxypeptidase and acts to remove dipeptide from the C-terminal of angiotensin I (decapeptide), producing angiotensin II (octapeptide), which is a vasoconstrictor (129). High levels of angiotensin-II result in undesirable blood vessel contraction that leads to the development of hypertension (128,129). ACE also inactivate bradykinin, a potent vasodilator leading to further inability of blood vessels to relax properly after contraction (130). Management of hypertension currently focuses on ACE inhibitors, angiotensin II receptor blockers, and diuretics. Synthetic inhibitors, such as captopril, enalapril, alacepril, and lisinopril, are commercially available, but their use is restricted due to possible adverse effects, including cough, taste disturbances, and skin rashes (131) and reported side effects and impacts on the fetus if used during pregnancy (132,133).

Regardless of their lower activity compared to pharmaceuticals on a weight-to-weight basis, antihypertensive peptides are believed to present a safer and cheaper option

to synthetic drugs for the treatment of hypertension (107). Therefore, a vast body of research has been dedicated to the antihypertensive effects of bioactive peptides from a range of marine sources (134–142).

Peptides displaying ACE inhibitory properties have been found to consist of short amino acid sequences with tyrosine, phenylalanine, tryptophan, lysine, leucine, isoleucine, valine, and arginine as dominant amino acids (143). In addition, peptides containing hydrophobic amino acids are the most effective ACE inhibitors, especially those with proline in the C-terminal and positively charged amino acids (arginine and lysine) in the N-terminal (144). Consequently, the ratio of hydrophilic-hydrophobic amino acids in the peptide sequence is a critical factor in ACE-inhibitory activity due to the disruption of ACE active site access to peptides by hydrophilic amino acid residues (145,146). Researchers have revealed that ACE selects substrates or competitive inhibitors that contain aromatic amino acid residues such as tryptophan, phenylalanine, and tyrosine at their C-terminal tripeptide sequence as well as branched and aliphatic amino acids such as glycine, valine, leucine, and isoleucine at the N-terminal (147) indicating a substrate specificity. But it is also identified that ACE has a wider distribution and a substrate specificity (148).

Yathisha et al., (149) reviewed the antihypertensive activity of fish protein hydrolysate peptides and found that enzymatic hydrolysis of fish proteins yields ACE inhibitory peptides exhibiting antihypertensive activity tested *in vitro* and *in vivo*. Similarly, the review by Ejike et al., (150), indicated the presence of a variety of antihypertensive peptides from microalgal sources, particularly those derived from *Chlorella* sp. Tejano et al., (67) isolated proteins from *Chlorella sorokiniana* and predicted potential peptides with biological activities using in silico analysis and suggested

that proteins of *C. sorokiniana* have high occurrence of bioactive peptides such as angiotensin-I converting enzyme (ACE) inhibitor, antioxidant peptides.

2.7. Summary

Bioactive peptides positively impact physiological systems and may ultimately have utility in preventative management of health and well-being beyond their nutritional capabilities. Microalgae and fish by-products are two readily available sources for extracting proteins and peptides economically and in an environmentally sustainable manner for the generation of bioactive peptides. Fish by-products and microalgae can be used as sources of bioactive peptides due to their high protein contents. Utilization of these sources of bioactive peptide production has positive environmental benefit would decrease waste. The variation in the amino acid composition of different protein hydrolysates depends on several factors such as raw material, enzyme source, and hydrolysis conditions. Although different sources may result in different bioactive peptides due to the inherent variations in the source (amino acid composition in the proteins), and hydrolysis production method, it is clear that a range of peptides can exhibit bioactivity in several functionalities. However, to produce these peptides for comparative analysis, extraction methods of peptide hydrolysates vary depending on the nature of the parent material. *Chlorella sorokiniana*, require more than a mild fermentation to break down to access the intra cellular protein materials because of the challenging cell wall. Therefore, a direct enzymatic method coupled with a disruptive microwave treatment to break down cell walls was selected as the method of algal hydrolysate production.

Chlorella sorokiniana biomass may produce bioactive peptides with similar profile and bioactive functionality to Trout bioactive peptides and may be complementary or additive. Similarity or synergy of function may help overcome the supply limitations of marine biomass by having a comparable high-value peptide stream but to biologically produce marine-like peptides that can serve the need for a range of functional ingredients in the food that can provide a variety of health benefits.

Furthermore, these peptides may have synergistic or complementary characteristics, which can be highly favorable in their applications as functional ingredients. For example, a peptide fraction with higher metal chelation, radical scavenging, and electron donor properties can be a perfect active ingredient. Therefore, such products can be sought with peptides that have synergistic or complementary characteristics.

Literature studies have identified antioxidant and antihypertensive peptides in both Trout and *Chlorella* species. This study focuses on comparing and characterizing the peptides produced through hydrolysis of Rainbow Trout (*Oncorhynchus mykiss*) by-products and *Chlorella sorokiniana*, for their comparative capacity to show antioxidant and anti hypertensive activity and assess the ability of algal peptides to be used as a supplementary resource for fish by-products.

A true side by side comparative within the same study has not been performed to determine compatibility or similarity of the functionality of source for algal and fish by-products. This study will open new avenues for new commercial applications as potential complementary supplements comprising two protein sources to generate balanced bioactive peptide health products. It is anticipated that these could provide complementary

or comparable bioactivities such as antioxidant and antihypertensive properties in the resulting hydrolysate and peptide fractions.

CHAPTER 3: MATERIALS AND METHODS

3.1. Materials

Powdered and freeze-dried *Chlorella sorokiniana*, was supplied by Marine Research Station, National Research Council, Ketch Harbour, Nova Scotia, Canada. Rainbow trout (*Oncorhynchus mykiss*) by-product was procured from Waycobah Trout processors (Whycocomagh, Nova Scotia, Canada) (harvested fish processed to HOG (Head on Gutted)). The following chemicals were obtained from Sigma-Aldrich: Flavourzyme (Protease from *Aspergillus oryzae*) ≤ 500U/g, Trypsin, ACE Activity Assay kit (Catalog Number CS0002), Acrylamide, potassium ferricyanide, GSH standard curve, $FeCl_2$, FerroZine reagent, 3-(2-Pyridyl)-5, 6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate. The following chemicals were obtained from ThermoScientific: Pierce Modified Lowry Protein Assay Kit, O-phthalaldehyde reagent, 5X Laemmli buffer, Coomassie Brilliant blue R250, Trichloroacetic acid (TCA), $FeCl_3$, EDTA, DPPH solution, Ethanol 99.5%, Sodium phosphate buffer (NaH_2PO_4, Na_2HPO_4),

Ultrafiltration membranes for use in tangential flow systems were obtained from Pall Corp., ON, Canada. Deproteinized whey 5% (wt/wt), (80–90% lactose) was obtained from Saputo, Inc., Montreal, Canada. Lactic acid bacteria inoculum (LAB, *Lactobacillus plantarum* CNCM MA 18/5U (>1 × 10^{10} cfu/g), *Pediococcus acidilactici* CNCM MA 18/5U (>1 × 10^{10} cfu/g)) was obtained from (LALFEED® LACTO, Lallemand Inc., Aurillac, France), C-18 SPE cartridge (HyperSepTM), from Thermofischer Scientific. All other chemicals used were analytical grade.

3.2. Production of bioactive peptides

Chlorella sorokiniana and Rainbow trout (*Oncorhynchus mykiss*) were used to generate bioactive peptides using the following methods. To generate protein hydrolysates, the Rainbow trout by-products autolytic fermentation method was utilized. In order to produce peptide hydrolysates from *Chlorella sorokiniana*, with a challenging cell wall, access to the intra cellular protein materials was achieved through a direct enzymatic method coupled with a disruptive microwave treatment. Both methods were selected to be gentle, green chemistry methods targeting maximum retention of the functionality of extracted peptides.

3.2.1. Rainbow trout (*Oncorhynchus mykiss*) by-product fermentation

The obtained Rainbow trout by-products (heads, frames, and viscera) were thawed at room temperature, mixed in a 1:1:1 ratio by weight, and blended (Ninja® Professional Blender BL660C, Shark Ninja Operating LLC, QC, Canada) to form a uniform semi-solid mix. A method similar to Rajendran et al., (151) was used. Deproteinized whey 5% (w/w) (80-90% lactose), and 1% (w/w) lactic acid bacteria inoculum were mixed thoroughly with the ground by-product mix and transferred in to a 1L bioreactor batch fermenter. The temperature in the batch fermenter (New Brunswick ™ Bioflow®/CelliGen® 115, GMI Inc., MN, USA) was maintained at 37 °C and agitated continuously at 300 rpm. pH was monitored using the online pH monitor in 1 min intervals and recorded in the batch fermenter. Samples were withdrawn at 0, 6, 24, 48, and 72 h and were stored at −20 °C for further analysis. Next, samples were centrifuged at 3600 rpm for 15 minutes. Fractions

were separated after freezing, and retained hydrolysate was stored at -20 °C for further analysis. A sample from the emulsion fraction was frozen and freeze-dried. Then, a freeze-dried emulsion sample was defatted by washing with 5% (w/v) (suspension) with petroleum ether 3 times and then dried at room temperature. Non-defatted and defatted emulsion samples were used in the analysis.

3.2.2. Microalgae (*Chlorella sorokiniana)* combined microwave and enzyme treatment.

An initial algae suspension of 10% (w/v) was prepared with mixing distilled water (pH 6.5). The algal suspension (600 mL) was microwaved using a microwave digestion system (MARS 6 230/60, CEM Matthews, NC, USA). A time period of 30 mins and a temperature of 50 ^{0}C were used in the microwave treatment. Enzymatic hydrolysis was carried out with the enzyme Flavourzyme similar to Tejano et al., (85) with modifications to time and enzyme concentration; enzyme was mixed with the slurry in an enzyme-to-substrate ratio of 1:100 (w/w). The slurry was cooled to 37 °C before hydrolysis. pH was monitored and maintained at 7.0 with the addition of 0.1 M NaOH, and hydrolysis was carried out at 37 °C and agitated continuously at 300 rpm. Enzymatic proteolysis was carried out for a period of 6 h. At the end of 6 h, the reaction was stopped by heating the mixture in a water bath at 95 °C for 5 min to deactivate the enzyme. The mixture was allowed to cool to room temperature and centrifuged at 3600 rpm for 15 minutes; then supernatant hydrolysate was stored at −20 °C until further processing. Samples were withdrawn at 0, 0.25, 0.5, 1, 2, 4, and 6 h and heated in a water bath at 95 °C for 5 min, allowed to cool to room temperature, then centrifuged at 3600 rpm for 15 minutes, and the supernatant hydrolysate was stored at −20 °C for further analysis.

3.3. Characterization of the hydrolysis processes

3.3.1. Determination of lactose and lactic acid concentration during trout by-product fermentation

The samples collected during the fermentation at 0, 6, 24, 48, and 72 hours were analyzed using High Performance Liquid Chromatography (HPLC) (PerkinElmer, Shelton, CT, USA) to estimate the lactose and lactic acid present in all trout by-product fermentation. The collected samples were diluted at 5× in nano pure water and centrifuged at 12,700 rpm for 20 minutes at 20 ^{0}C temperature. ~800 µL of supernatant transferred to new centrifuge tubes and kept refrigerated. Samples were filtered with 0.45 µm syringe filter into a HPLC vial. The chromatographic column used for separation of compounds was Aminex HPX-87H (300 × 7.8mm), an organic acid column (Bio-Rad Laboratories, CA, USA). The standard peak area compound identification and quantification of lactose and lactic acid was performed at concentration 2mM – 100mM and 2mM- 300mM, respectively. The diluted samples (20 µL) were eluted in an isocratic mode with 0.0008N H2SO4 solution at 0.6 mL/min flow rate at 35°C.

3.3.2. Lactic acid bacteria growth

Inoculum growth over the course of trout by-product fermentation was determined by colony counting on DeMan-Rogosa-Sharpe (MRS) agar (Sigma-Aldrich, MO, USA) plates. The the liquid fraction of samples collected during the fermentation at 0, 6, 24, 48, and 72 hours were serially diluted (x 10^{-5}) in 0.9% sodium chloride (NaCl) solution and spreaded (100 µL) on the MRS agar plates. The plates were incubated for 48 hours at 37

^{0}C after which colonies were counted and expressed as colony forming units per mL (cfu/mL).

3.3.3. Proximate composition analysis

The proximate analyses were performed by following the methods described by AOAC (1990). The moisture content in Trout and *Chlorella* was determined by drying in a forced air convection oven at 105°C for 24 h. The ash content was determined by heating the samples in a muffle furnace at 500°C for 4 hours (AOAC, 1990). Lipid content was determined by Soxhlet extraction (SOXTHERM ®, C. Gerhardt UK Ltd, UK) using petroleum ether as extraction solvent (AOAC, 1990). The moisture content of the biomass was calculated by the weight removed by drying using the following equation.

$$\text{Moisture content (\%)} = 100 - \left(\frac{Sample\ weight\ after\ drying}{Sample\ weight\ before\ drying}\right) \times 100$$

The ash and lipid content was determined by weighing the sample left after the treatment using the following equation.

$$\text{Ash or lipid content (\%)} = 100 - \left(\frac{Sample\ weight\ after\ process}{Sample\ weight\ before\ process}\right) \times 100$$

The total N content was determined using 2 mg of oven-dried microalgae powder and fish by-product mix samples to determine the initial protein content. The samples were analyzed using an automatic analyzer Model PE 2400 Series II CHNS/O (Perkin Elmer, Norwalk, CT, USA) according to the manufacturer's instructions. 4.78 and 6.25 nitrogen to protein conversion factors were used for *Chlorella* and Trout respectively.

3.3.4. Soluble protein determination in hydrolysate samples

Samples were thawed and transferred to weighed tubes and centrifuged (3,600 rpm) for 15 min. The hydrolysis fraction was then collected into pre-weighed tubes and lyophilized. The freeze-dried supernatants were dissolved at 2 mg/ml concentration in a 0.1 M sodium phosphate buffer (pH 7). Pierce Modified Lowry Protein Assay Kit was used according to the manufacturer's instructions to determine the protein content of samples at 2 mg/mL concentration. Absorbance was measured using a microplate reader (Tecan M1000, Switzerland) at 750 nm and plotted on a bovine serum albumin standard curve to determine the protein content.

3.3.5. Degree of hydrolysis

The degree of hydrolysis was calculated for final hydrolysate samples and time point samples based on the method reported by Nielsen et al. (152). Sample (1 mg/mL, 33 µL) was added to 250 µL of O-phthalaldehyde reagent in the presence of dithiothreitol(DTT) forming a colored compound, and the absorbance was measured using a microplate reader (Tecan M1000, Switzerland) at 340 nm to detect the amount of primary amino groups present in the sample. 1.0 and 0.4 values were used as α and β values to calculate the degree of hydrolysis for both trout and *Chlorella* samples.

3.3.6. Molecular weight distribution analysis of hydrolysates

The protein compositions of the hydrolysate samples were characterized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Van Cauwenberghe et al. (2002), (153) with modifications. Samples were mixed with 2X Laemmli buffer (1:1 volume) and heated at 95°C for 5 minutes before

loading (20 µL) into the wells of a 6% (w/v) stacking gel for SDS-PAGE analysis. Proteins were separated using a 20% (w/v) resolving gel with an applied voltage of 100 V using a Tris glycine SDS running buffer. A protein-based molecular weight ladder was loaded with the samples to evaluate the molecular weight distribution of the hydrolysed proteins. After electrophoresis, the gels were stained using Coomassie Brilliant Blue R-250. The colored gel was destained using methanol 50% (volume), acetic acid 10% (volume) solution, and then visualized using Epson Perfection V800 scanner.

3.3.7. Fractionation of hydrolysates by peptide molecular weight

Final protein hydrolysate samples from the two source materials were fractionated according to a method described by Mohan et al., (154) with modifications. The selected protein hydrolysate sample was passed through a tangential flow system consisting of an ultrafiltration membrane with a molecular weight cut-off (MWCO) of 10 kDa (Pall Corp., ON, Canada). After 4 h, the resulting permeate (<10 kDa peptide fraction) was collected and passed through a 3 kDa MWCO membrane under similar conditions to recover the 1–3 kDa peptide fraction. The process was repeated with the 1kDa MWCO membranes to obtain the 3–10, 1- 3, and <1 kDa peptide fractions. The peptide fractions were freeze-dried and stored at -20 °C for further analysis.

3.3.8. Amino acid composition analysis

Amino acid analysis was carried out at SPARC BioCentre, The Hospital for Sick Children. Toronto, Canada. Defatted samples were subjected to vapor phase hydrolysis by 6N HCl with 1% phenol at 110°C for 24 h under pre-purified nitrogen atmosphere in pyrolyzed borosilicate tubes in a vacuum centrifugal concentrator (Eldex Laboratories Inc., Napa,

CA, USA). Pre-column derivatization was carried out at room temperature using phenyisothiocyanate (PITC) mixture along with 7:1:1:1 methanol, water, triethylamine, followed by resuspension in phosphate buffer. Phenylthiocarbamyl (PTC) amino acid mixtures were transferred to injection vials and run (2 µL injection) on a Waters Acquity System connected with a BEH C18 column for reverse-phase separation at 48 ^{0}C and detected at 254 nm.

3.4. Characterization of the bioactivities of fractionated hydrolysate samples

3.4.1. Angiotensin-converting enzyme inhibition

Antihypertensive properties of the fractionated hydrolysate samples were quantified by measuring the ACE activity according to the protocols provided by the ACE I Activity Assay kit with modifications. The assay was performed on 50 µL of lyophilized fractionated hydrolysate samples (1 mg/ml in water). Standards were diluted 10-fold to a final concentration of 100 µM: 10 µL of the Standard stock solution with 90 µL of Assay Buffer. The substrate was diluted 100-fold in Assay Buffer. 50 µL of sample and 40 µL of the diluted substrate were added to the sample wells. 50 µL of assay buffer and 40 µL of the diluted substrate were added to the positive control wells. 50 µL of standards and 40 µL of the diluted substrate were added to the standards wells. 96 well plate was taken to 37 ^{0}C for 5 minutes. The reaction was initiated by adding 10 µL of the diluted enzyme to the wells with standards, samples, and positive control, for a final reaction volume of 100 µL/well. Fluorescence was read after 5 minutes by setting the microplate reader (Tecan M1000, Switzerland) to excitation at 320 nm and emission at 405 nm. Positive control was

used as the uninhibited ACE activity to calculate the inhibition percentage of the samples calculated as percent inhibition.

3.4.2. Ferric reducing antioxidant capacity

The ferric reducing antioxidant potential of the fractionated hydrolysate samples was determined by using a method described by Mohan et al., (154) with modifications as follows. Equal volumes (120 µL) of the sample (2 mg/mL) and potassium ferricyanide (1% w/v) were mixed and incubated for 20 min at 50°C. TCA (10% w/v) was then added (120 µL) and vortexed before centrifuging the mixture at 8417 rpm. Distilled water (80 µL) and supernatant (100 µL) were transferred to a 96-well plate in triplicate. A 20 µL of $FeCl_3$ (0.1% w/v) was added to each well, and the plate was incubated for 10 min at room temperature. Then the absorbance was read at 700 nm using a microplate reader (Tecan M1000, Switzerland). A Reduced L-glutathione (GSH) standard curve (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, and 1 mM) was used to express the ferric reducing potential as mM GSH equivalent.

3.4.3. Fe^{2+} chelating ability

The metal chelating ability of the fractionated hydrolysate fractions was determined based on a method by Xie et al. (155) with minor modifications as follows. A 500 µl of sample (2 mg/ml in distilled water) was equilibrated with 25 µl of $FeCl_2$ (2 mmol/L) for 10 minutes at room temperature (RT). Then, 50 µl of FerroZine solution was added and incubated for another 10 minutes at RT. A 50 µl of the prepared mixture was transferred to a 96-well plate, and absorbance was measured at 562 nm using a microplate reader (Tecan M1000,

Switzerland). An EDTA standard curve (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, and 1 mM) was used to express the Fe^{2+} chelating potential as mM EDTA equivalent.

3.4.4. Radical scavenging ability

The DPPH radical-scavenging activity of the freeze-dried hydrolysate was determined according to the following method. A 200 µl of DPPH solution (0.1 mM in 99.5% EtOH) was mixed with 200 µl samples (2 mg/ml in pH 7, 100 mM sodium phosphate buffer), and the mixture was incubated for 30 minutes in the dark at room temperature. GSH was used as a standard (2 mg/ml) and 500 µl of 100 mM sodium phosphate buffer as blank. The solutions were centrifuged at 6,400 x g for 3 minutes, then pipetted (200 ul) into a 96-well plate in triplicate, and the absorbance was measured at 517 nm using a microplate reader (Tecan M1000, Switzerland) to determine the reduction of DPPH. The following equation was used to determine the DPPH radical scavenging activity: $(\%) = [(A_{blank} - A_{sample}) / (A_{blank} - A_{standard})] \times 100$.

3.4.5. Peptide sequencing of selected hydrolysate fractions

Samples for sequencing were selected based on exhibited overall antioxidant and ACE inhibition capacity. Proteomics analysis for the selected 1-3kDa fraction of trout and *Chlorella* samples was carried out at the SPARC BioCentre, The Hospital for Sick Children, Toronto, Canada. Desalted samples (5 µL; using Millipore Ziptip) were injected using an EASY-nLC 1000 nano-LC system interfaced to a Thermo Scientific Exploris 480 MS system. Runs were set for 60 mins with solvent A (0.1% formic acid (v/v)) and B (80% acetonitrile, 0.1% formic acid (v/v)) with a PepMax RSLC EASY-Spray column filled with 2 µm C18 beads (ThermoFisher, San Jose, CA) column. Proteomic analysis of mass spectra

was carried out using PEAKS Studio 10.6 build 20201221 software. Database search was carried out against uniprot_UP000193380_Oncorhynchus_mykiss_26072021 and uniprot_UP000239899_*Chlorella_sorokiniana*_ 26072021. The following parameters were used with the database search: parent tolerance: 50.0 ppm, fragment tolerance: 0.02 Da, enzyme: unspecific cleavage, fixed modifications: +57.02 Da on C (carbamidomethyl), variable modifications: +15.99 Da on M (oxidation), +0.98 on N, Q (deamidation), +42.01 on peptide N-terminal (acetylation).

3.5. Statistical analysis

All the analyses were conducted in triplicates, and the results were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was performed for biological activity (antioxidant and ACE inhibition) analysis followed by Tukey's HSD test for the separation of means using Minitab 19 statistical software (Minitab Inc., USA) at a 95% confidence interval ($\alpha = 0.05$).

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Trout and *Chlorella* chemical composition

The Trout by-product mix and *Chlorella sorokiniana* composition are shown in Table 3. Rainbow trout by-product mix had a higher lipid content, 57.73% dw (21.51% wet weight) as compared to literature reports for eviscerated fish (10.48% ww) and trout muscle (4.43% ww) (156,157). This significant variation in lipid may be due to the ratio of viscera to frames and heads in the product samples used and associated high lipid content of salmonid liver and subcutaneous lipid. Literature values for whole-body rainbow trout protein content are around 19% (ww), higher than the observed value (16.24% ww) for the by-product mix observed here. This likely reflects the lack of muscle protein in the by-products mix used for this research.

Table 3: Chemical Properties of rainbow trout Vs *Chlorella sorokiniana* (g/100 g dw)

Parameters	Rainbow trout	*Chlorella sorokiniana*
Ash	6.19± 0.53	12.27± 0.43
Fat	57.73± 1.87	0.44±0.69
Protein	29.81± 0.64	31.77±0.14

Previous literature analyses of *C. sorokiniana* dry weight show that the species is composed of 40% protein, 30–38% carbohydrate, and 18–22% lipid (158–160). However, in this research, the observed lipid content for *C. sorokiniana* is very low compared to the previous results reported in other studies, while protein is slightly lower (30.33%). Reasons for this variation could be harvesting time, cultivation methodology, and subspecies of natural differences.

4.2. Trout fermentation

4.2.1. pH change

Figure 2 depicts the pH evolution across the processing period for trout by-product mix, which had an initial pH value of 6.5. At the maintained temperature (37 ^{0}C), the pH of the microbial-assisted fermentation gradually decreased over 72 h of the bioprocessing period. This was attributed to the gradual lactic acid production through bioconversion of lactose by the two inoculant LAB strains *L. plantarum* and *P. acidilactici*. (Figure 4). The pH stabilized after 48 h and remained constant at an average value of 4.3. Attainment of this pH value was considered a satisfactory completion point for the hydrolysis since the growth of spoilage microorganisms is inhibited within this pH range (151), and acidic hydrolysis is anticipated to be complete (Figure 6 – Degree of Hydrolysis of the hydrolysate fractions at different time points of Trout fermentation). The pH curve from the present study is comparative to previous reports for LAB fermented fish waste (151,161)

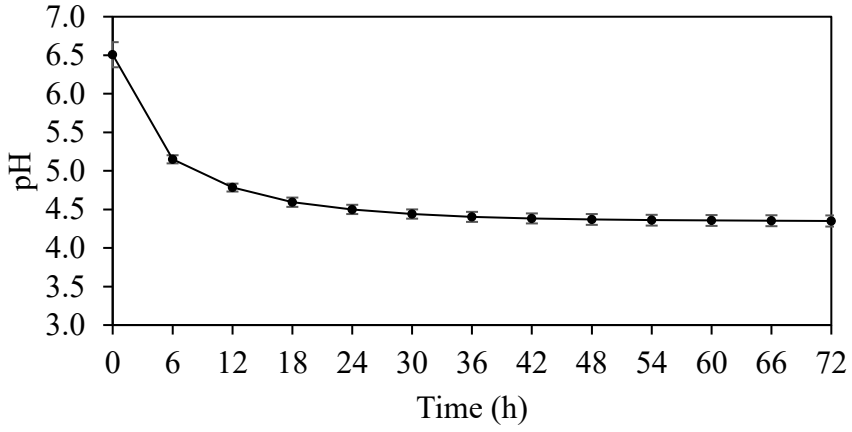

Figure 2: Change in pH of microbial-assisted hydrolysis of Trout by-product mix during 72 h processing time

4.2.2. Lactose utilization and lactic acid production

Figure 3 depicts the change in lactose concentration in fermentation media during the LAB fermentation. There is a noticeable rapid reduction in lactose concentration during the first 24 h from ~120 mM to ~20 mM, the first 6 h being the highest rate of consumption. After the first 24 h, lactose concentration continued to decrease but at a diminishing rate of decrease resulting in a final concentration of 10.2 mM at the end of the fermentation achieving a ~92% utilization.

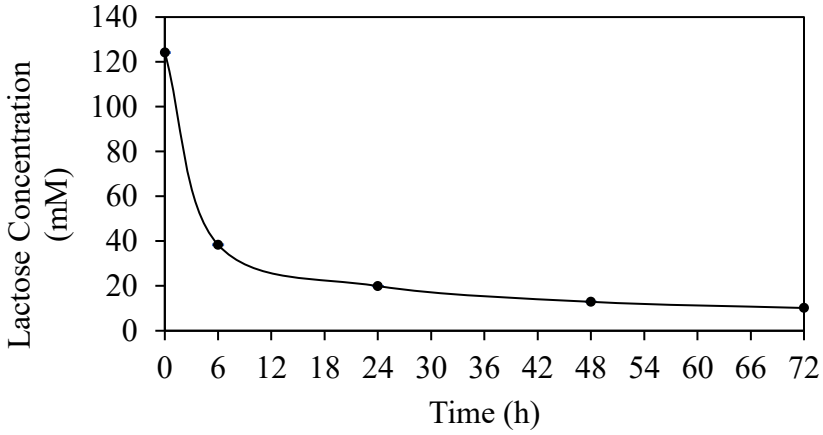

Figure 3: Change in lactose concentration in system during the trout by-product mix fermentation period

Figure 4 depicts the change in lactic acid concentration during the trout by-product mix fermentation period. It is observed that more than 50% of the increase in lactic acid concentration occurs in the first 24 h. But the increase in lactic acid concentration is not directly correlated to the reduction rate of lactose from the fermentation system. This may be due to the lactose utilization for cell growth in the first 6 h, which is depicted in figure 5. After the first 24 h, the rate of lactic acid production declined to reach a maximum

concentration of 147 mM. This reduction is attributed to substrate depletion and decline in LAB population due to rising osmotic pressure and decreasing pH in the system.

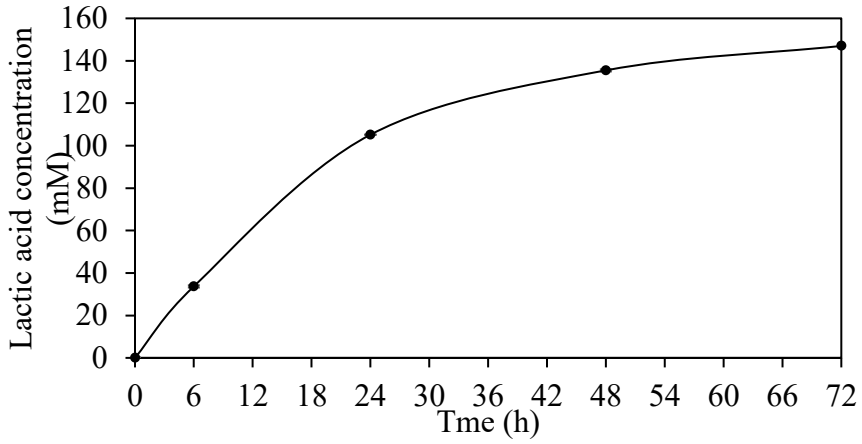

Figure 4: Change in lactic acid concentration in system during trout by-product mix fermentation period

4.2.3. Microbial counts

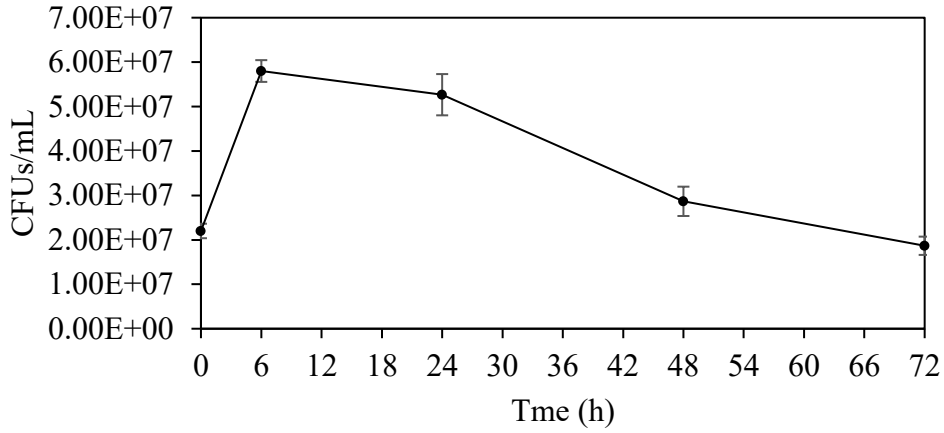

Figure 5: Change in Lactic acid bacteria growth during Trout by-product mix fermentation period

Figure 5 depicts LAB growth throughout the fermentation period at an incubation temperature of (37 °C). Microbial LAB populations showed a sharp and significant increase within the first 6 h, followed by a drop. The maximum cell count was observed at 6 h, while the minimum counts obtained at 72 h coincides with the lowest pH. It was observed that the highest drop in pH occurred in the first 6 h, a drop from 6.5 to 5.15. Subsequently, the drop in pH may have negatively affected the LAB growth resulting in a declining LAB population. Similar microbial growth profiles were observed for redfish and salmon hydrolysates prepared using lactic acid bacteria (151,161). At the same time, there is a significant (more than 50%) drop in residual lactose, also observed (figure 3), which could also lead to reduced microbial growth. As with previous studies, it is clearly observed that LAB inoculum was more sensitive to pH change due to lactic acid production. In fact, pH change has been found to affect the growth characteristics (either decreases or stops the growth) of large lactic acid bacteria (162). Due to the lipophilic nature of organic acids, protons (H^+ ions) believed to be diffused into the plasma membrane causes inhibition of major metabolic activities in the cells (162,163).

4.2.4. Degree of hydrolysis

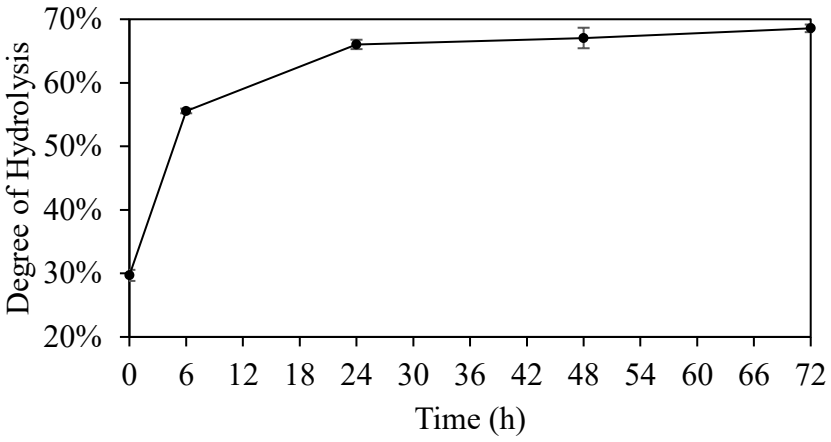

Figure 6: Change in degree of hydrolysis of the hydrolysate during Trout by-product mix fermentation period

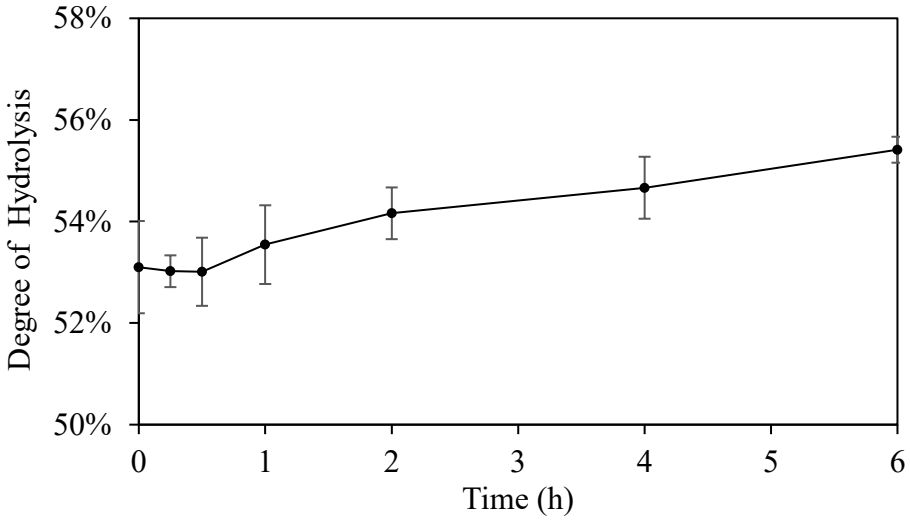

Figure 7: Change in degree of hydrolysis of the hydrolysate during *Chlorella* enzymatic treatment period

The hydrolysis kinetics of the microbial-assisted fermentation and *Chlorella* enzymatic treatment was studied across the entire processing period by taking out samples at different time points. The degree of hydrolysis, which represents the ratio of the number of peptide bonds cleaved with respect to the total number of bonds per unit weight, is one of the most important parameters governing the protein hydrolysate utilization. The OPA method used in this study is based on the reaction between OPA and primary amino groups, which produce 1-alkylthio-2-alkyl-substituted isoindoles in the presence of thiol. The isoindoles can be quantified spectrophotometrically at 340 nm. When peptide bonds break down, the amount of primary amino groups in the solution increases.

Figure 6 depicts the increase of the degree of hydrolysis of Trout by fermentation at different time points. Figure 7 depicts the increase of the degree of hydrolysis of *Chlorella* using enzyme treatment at different time points. Trout hydrolysate achieved 68.6% hydrolysis at the end of the 72 h, and the highest increase was recorded in the first 24 h. Literature data utilizing a similar process for Atlantic salmon (*Salmo salar*) indicated a similar trend, achieving the highest level of degree of hydrolysis on the 4^{th} day of the treatment at around 55%, with the highest increase recorded during the first 24 h (151). In another study, trout by-product hydrolysates generated using trout pepsin reported 30% hydrolysis after 5 h of treatment (164). However, it is noted that different enzyme formulations behave differently on producing hydrolysates resulting in various degrees of hydrolysis (165).

Chlorella hydrolysate achieved 55.4% hydrolysis at the end of the treatment (Figure 8). Similar results (55.76% degree of hydrolysis) were reported in a study (166) carried out to optimize enzymatic hydrolysis for the production of antioxidative peptides

from *Nannochloropsis gaditana* using Alcalase enzyme treatment. However, other studies have reported around ~20% degree of hydrolysis in general when microalgal biomass is treated with different enzymes (167,168). One reason for this lower DH could be the method used in determining the DH. It is identified that high exopeptidase activities under-estimate DH in pH-stat method (release of protons during hydrolysis into the medium leads to a reduction in the pH of the reaction mixture. The number of peptide bonds cleaved can be estimated from the amount of base required to maintain a constant pH during the reaction) used in both studies (169). The current research observed that the initial degree of hydrolysis was higher at the start of the enzymatic treatment, perhaps due to the microwave pretreatment and prior processing after cultivation (freezing and freeze-drying). It is reported that microwave pre-treatment helps disrupt the cell wall and membrane, enabling the release of cell contents and facilitating the access of enzymes to the cytoplasm (170).

4.2.5. Molecular weight distribution (protein composition change)

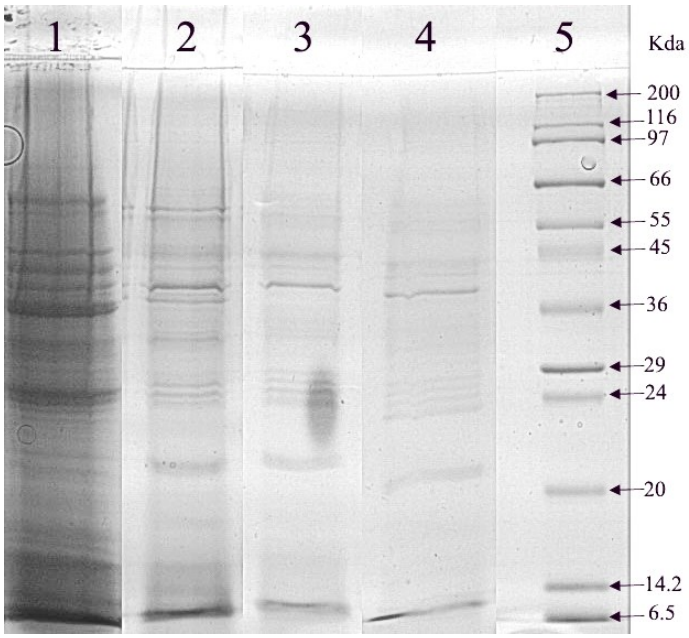

Figure 8: SDS-PAGE profile of Trout by-product hydrolysate prepared through fermentation. Lane 1: 6h; Lane 2: 24h; Lane 3: 48h; Lane 4; 72h; Lane 5: molecular weight markers

The SDS-PAGE protein hydrolysate patterns of Trout and *Chlorella* throughout the treatment period (Figure 8 and 9) showed that protein digestion occurred in both Trout by-product mix fermentation and *Chlorella* enzymatic treatment. High molecular weight proteins evident initially in trout by-products (Figure 8 Lane 1) were degraded into smaller peptides as the fermentation hydrolysis progressed. The disappearance of bands in the high molecular weight region was observed toward the end of the fermentation period. The combination of a small change increase in the degree of hydrolysis value and lack of changes in molecular weight profile in samples at 48h may indicate the completion of trout by-products hydrolysis by 48 h. Inherent enzymatic breakdown of proteins/peptide

fractions may decline due to loss of enzyme activity or the reduction in accessible peptide bonds available for breakdown (171,172).

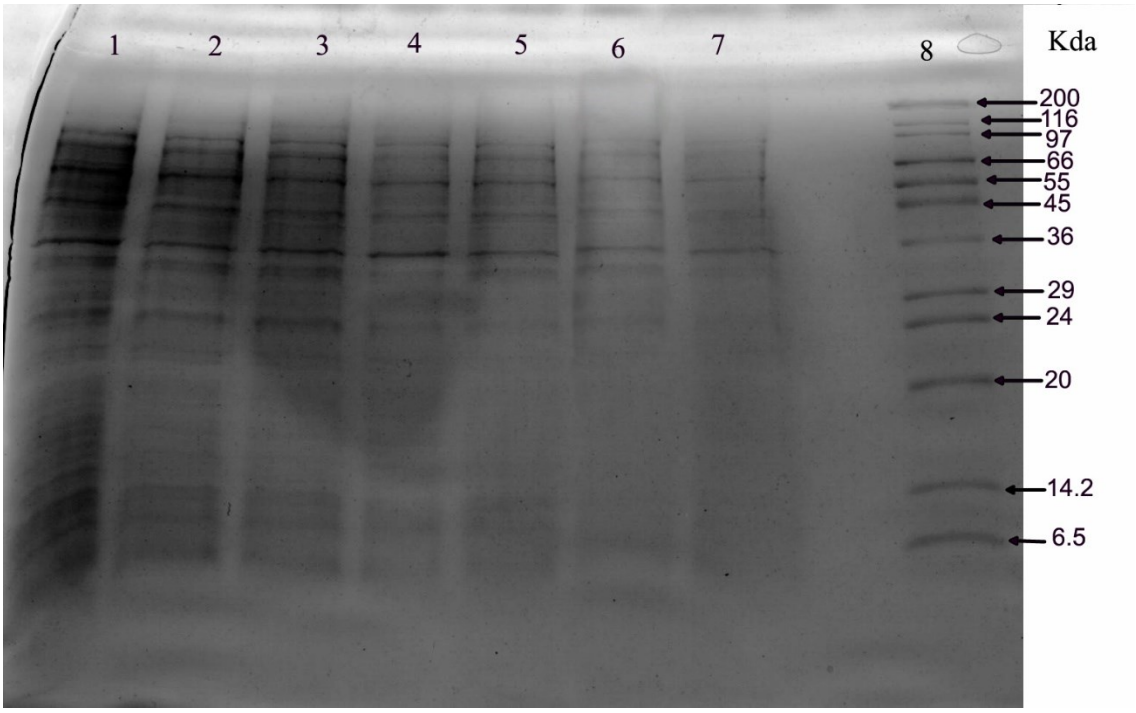

Figure 9: SDS-PAGE profile of Chlorella hydrolysate prepared through *Chlorella* enzymatic treatment. Lane 1: 0 min; Lane 2: 15min; Lane 3: 30min; Lane 4; 1h; Lane 5: 2h; Lane 6: 4h Lane 7: 6h; Lane 8: molecular weight markers

Several muscle proteins were anticipated to be present in trout by-products (Figure 8 Lane 1). Protein bands corresponding to myoglobin (~17kDA), tropomyosin (~36kDa), and actin (~42kDa) were observed. However, no bands about predicted high molecular weight proteins were detected which could be due to the enzymatic degradation during storage and processing (173). Similar results were reported in a previous study that depicted redfish's intact proteins (*Sebastes mentella*) degraded into smaller peptide fractions using the same conditions but over a 6-day fermentation.(161).

In microwave pretreated *Chlorella* biomass, high molecular weight proteins (greater than 670 kDa- complex soluble aggregates of proteins and chlorophyll) (Figure 9 Lane 1) were degraded into smaller peptides as the enzymatic hydrolysis progressed. However, as depicted in the degree of hydrolysis values (Figure 7), there was a relatively small increase in the degree of hydrolysis observed during the treatment time. The resulting hydrolysates have mixed peptides, as shown in the gel, having no distinct protein bands. The disappearing bands (116-45KDa) affirmed that enzymatic hydrolysis effectively degraded the available proteins into smaller peptides. There were no chlorophyll a-b binding proteins apparent in the hydrolysate and usually present in the range 88 and 28 kDa (174), a largely abundant protein located in the thylakoid membranes of chloroplast (175). These proteins signal the amount of cell breakdown (autolysis), providing access to other protein(s) to solubilize in the medium (176). The results also showed that the proteins present in the hydrolysate were highly diverse, which may be clarified by the fact that microalgae do not have specific storage protein as their nitrogen source, which accumulates in both vegetative and reproductive tissues in plants and serve as a reservoir to be used in later stages of plant development (177).

4.2.6. Fraction yield

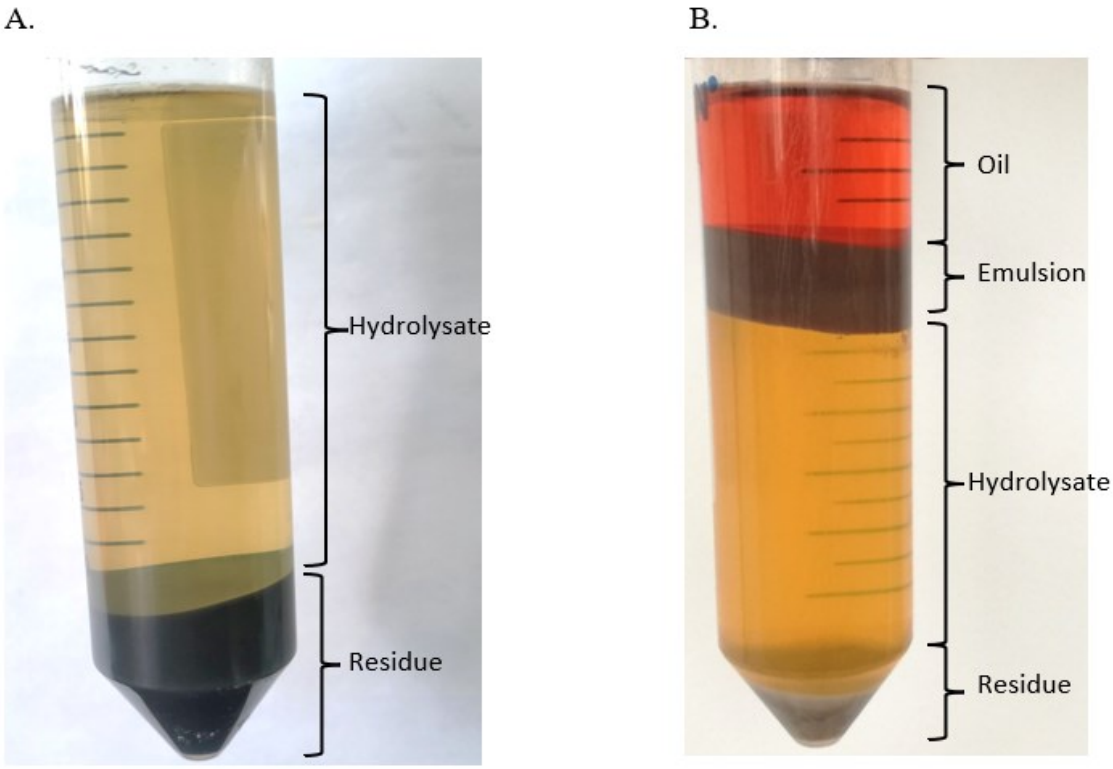

Figure 10: Different fractions obtained after centrifugation of hydrolysate samples. A: *Chlorella* treatment; B: Trout fermentation

Figure 10 depicts centrifugation of final hydrolyzed *Chlorella* product and trout by-product mix fermentation product yielding two and four distinct fractions, respectively. Table 4 shows the different amounts obtained of different fractions on a wet weight basis for trout fermentation. Rajendran et al., (151) reported around 50-60% of hydrolysate yield using the same methodology for Atlantic salmon visceral by-products. In this study, ~18.5% oil fraction was obtained. This indicates the high oil content in the by-products, as identified in the proximate composition analysis (Table 3).

Table 4: Yield of different fractions at the end of centrifugation of trout fermentation and *Chlorella sorokiniana* treatment (wet weight percentage)

Fraction	Rainbow trout	*Chlorella sorokiniana*
Hydrolysate	52.66 ± 1.20	45.33 ± 2.73
Oil	18.56 ± 0.87	-
Emulsion	17.26 ± 0.29	-
Residue	7.75 ± 1.08	53.61 ± 2.37
Losses	3.76 ± 1.67	1.06 ± 0.63

Table 4 shows the different amounts obtained of different fractions on a wet weight basis for pretreated, hydrolyzed *Chlorella*. The hydrolysate fraction yield was ~45% of total hydrolysate at the end of the centrifugation of the treated biomass. There were two distinct fractions (hydrolysate and residue) that were noticeably identified. There was no apparent separation of oil or emulsion fraction identified, which indicates the low fat/ lipid content (~0.44%) as identified in the compositional analysis. The residue fraction was shiny and slimy, shown in Figure 11.

Figure 11: Residue fraction of *Chlorella* treatment after centrifugation of *Chlorella sorokiniana* treatment

4.2.7. Protein concentration and amino acid composition of hydrolysates

After hydrolysis treatments, and separation hydrolysate fractions were evaluated for protein concentration using a modified Lowry assay. *Chlorella* hydrolysate contained 4.96 ± 0.181 mg/mL protein concentration, while for the trout, this was 40.04 ± 2.5734 mg/mL. There was a significant ($P < 0.05$) difference between the two protein concentrations, which indicated the microalgal biomass's low protein digestibility or solubility. The cell walls of microalgae are one of the biggest challenges in accessing and hydrolyzing cellular proteins, which appears to be the case for *Chlorella*. Observed protein concentration results also indicate the apparent ineffectiveness of the microwave pre-treatment used for the current process to extract proteins from the *Chlorella* biomass. A study conducted with *Chlorella sorokiniana* utilizing alkaline extraction followed by isoelectric point precipitation to generate protein isolates resulted in a 65.08 % (wt/wt initial biomass dry basis) protein content which is higher than the *C. sorokiniana* biomass protein content which was at 58.23% (wt/wt initial biomass dry basis) (178). This indicates that it is possible to extract high concentrations of protein hydrolysates from *Chlorella spp* utilizing harsh alkaline methods.

For Salmonid by products, Rajendran et al., (151) reported a similar (~40%) hydrolysate protein content using Atlantic salmon visceral by-products in a lactic acid fermentation after day 2 with no significant change reported at the end of the treatment (day 8). This is in agreement with the observed results of this research. By comparison, the same study reported 60% protein concentration attainment at day 2 and 80% protein concentration at day 4 using a formic acid treatment. Therefore, it is clear that there are

clear differences between chemical treatments and the mild biological treatments used here on the protein concentration of the final hydrolysate.

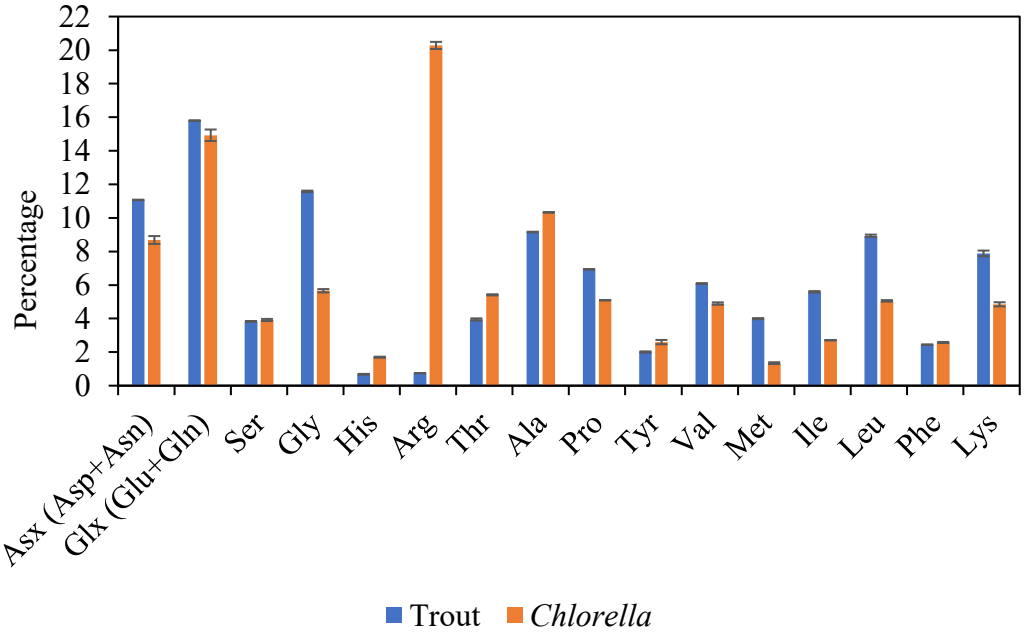

Figure 12: Comparison of amino acid composition of trout by-product mix and *Chlorella sorokiniana* hydrolysate.

The most abundant amino acids in rainbow trout by-products were glutamine and glutamic acid, glycine, asparagine, and aspartic acid, alanine, and leucine, which accounted for 15.8%, 11.58%, 11.07%, 9.16%, and 8.93%, respectively (Figure 12). These results are comparable to previously reported results. Taheri et al., (179) reported glycine, glutamine or glutamic acid, asparagine or aspartic acid, and proline as the most frequently occurring amino acids from rainbow trout viscera hydrolysate using alcalase enzyme digest. Suwal et al., (47) reported glycine (>30%), proline, alanine, and glutamic acid as the most abundant amino acids found in the antioxidant peptide fraction of molecular mass < 30 kDa isolated from rainbow trout skin gelatin hydrolysates.

Hydrolysate from *Chlorella* had the most predominant amino acid composition comprising arginine, glutamine or glutamic acid, alanine, asparagine or aspartic acid, and glycine, accounting for 20.28% 14.93%, 10.33%, 8.68%, and 5.66%, respectively (Figure 12). These results were comparable to the reports of Tejano et al., (85) on *Chlorella sorokiniana*, Ursu et al., (180), and Morris et al., (167) on *Chlorella vulgaris* except for the arginine content which was higher in the current study. Tejano et al. (85) reported that aspartic acid, glycine, alanine, and proline were the most abundant amino acids in *Chlorella sorokiniana* protein isolates enzymatically hydrolyzed using pepsin, bromelain, and thermolysin, with glutamic acid values being the highest. In contrast, this research produced hydrolysate with the highest content amino acid of arginine for *Chlorella sorokiniana*.

4.3. Bioactivities of molecular weight fractions and hydrolysates

4.3.1. Ferric reducing antioxidant capacity

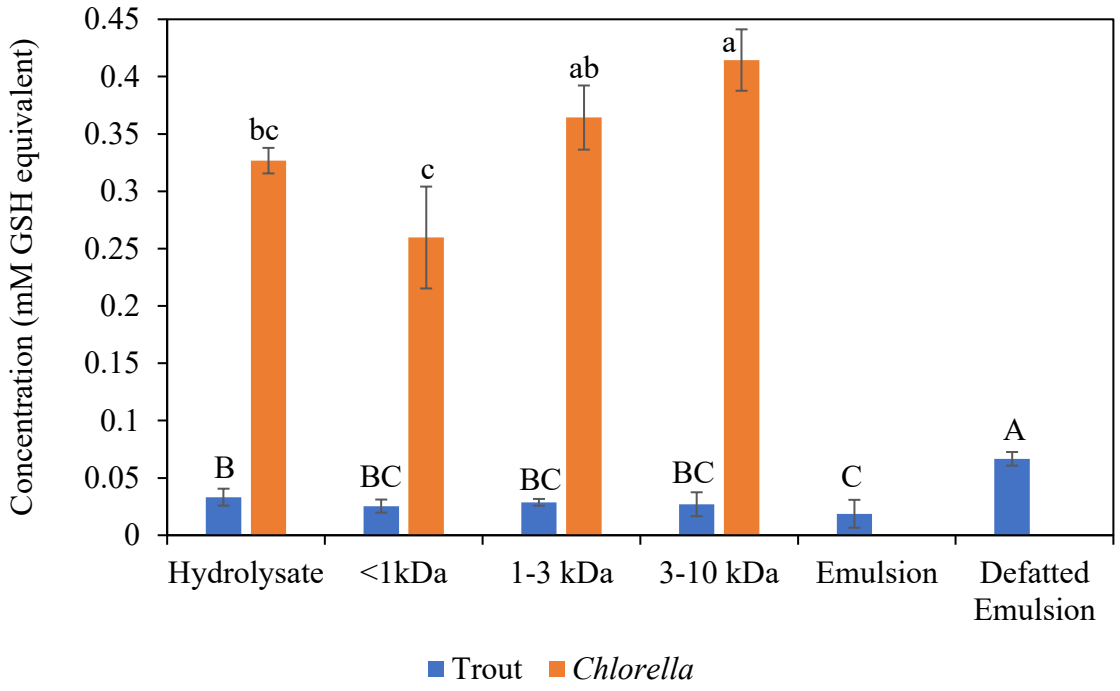

Figure 13: Ferric reducing power of lyophilized trout, *Chlorella* hydrolysates, molecular weight fractions, and trout emulsion and defatted emulsion at 2mg/mL concentration. *Note:* Bars (mean ± standard deviation, n = 3) with different alphabets have mean values that are significantly different at $p < 0.05$.

The ferric reducing antioxidant capacity assay is used to evaluate the ability of a potential antioxidant to donate an electron to the free radical (181). Samples with higher reducing power have better abilities to donate electrons. The reducing powers of both lyophilized hydrolysates, as well as the trout emulsion fraction and defatted emulsion fractions, are shown in figure 13. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each hydrolysate. The presence of reducers causes the reduction of the Fe^{3+}/ferricyanide complex to the

ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm indicates the Fe^{2+} concentration (182). The reducing power of the protein hydrolysates and peptide fractions was varied. *Chlorella* hydrolysate and its MWCO fractions demonstrated significantly higher ferric reducing capacity than the trout hydrolysate and its MWCO fractions. Among the trout hydrolysate samples, defatted emulsion produced the highest ferric reducing capacity, while in the case of *Chlorella* the highest reducing power activity was exhibited by 3-10 kDa fraction and secondly by the 1-3 kDa fraction of *Chlorella* hydrolysate. In prior studies, larger peptide fractions of *Chlorella* showed better reducing power than smaller peptide fractions, and a similar trend was observed in the current study in the case of *Chlorella* hydrolysate (178,183). In contrast, the current study found no significant differences at $p < 0.05$ in the reducing power activities of trout hydrolysate samples of differing MWCO. Peptides that have high absorbance demonstrate higher reducing power caused by the ability to donate hydrogen or electron. A high concentration of hydrogen ions may increase the reducing power in the hydrolysate and their MWCO fractions. Therefore, the presence of amino acids such as histidine, leucine, isoleucine, lysine, tyrosine, and methionine with increased availability of hydrogen ions in the protein hydrolysates and peptide fractions may have influenced their reducing power activities (183). These results showed that all protein hydrolysates and peptide fractions could donate electrons and reduce agents to differing degrees.

The results suggest that the *Chlorella* protein hydrolysates and isolated molecular weight fractions have high reducing power and be used as reducing agents. However, differences between these and literature processing and strain differences regarding reducing powers of the protein hydrolysates and peptide fractions may have been

influenced by the combination of microwave and enzymatic treatment used for the hydrolysis, which could produce peptides with varying compositions, sequences, and sizes.

4.3.2. Fe^{2+} chelating ability

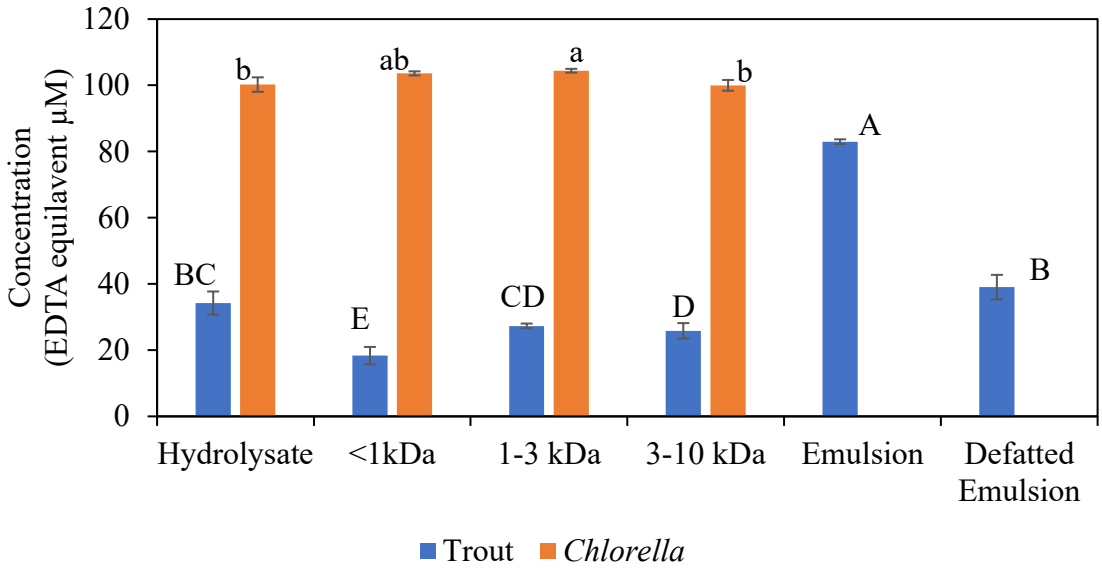

Figure 14: Ferrous chelating ability of lyophilized trout, *Chlorella* hydrolysates, molecular weight fractions and trout emulsion and defatted emulsion at 2mg/mL concentration. *Note:* Bars (mean ± standard deviation, n = 3) with different alphabets have mean values that are significantly different at $p < 0.05$.

Transition metal ions, for instance, Fe^{2+}, can catalyze the generation of ROS that promote oxidative damage to critical cellular compounds such as DNA and proteins that can lead to diseases (184). Therefore, the chelation of these transition metal ions by antioxidative peptides could prevent or reduce the negative effects of the Fe^{2+}-catalyzed generation of ROS. Figure 14 illustrates the ability of hydrolysate and molecular weight fractions generated in this study to chelate Fe^{2+}. In trout samples, the emulsion fraction exhibited the strongest chelating capacity while low MW fractions (<1 kDa to 3-10kDa) possessed weaker Fe^{2+} chelating activity ($p < 0.05$) with <1kDa fraction exhibited the

lowest Fe^{2+} chelating ability overall. Similar results were observed by Tang, Wang, and Yang (185) on hemp protein hydrolysate, which report that longer peptide chain length could lead to higher iron-chelating effects. However, there was no significant difference in Fe^{2+} chelating ability among the *Chlorella* samples observed with the change in MWCO as with ferric reducing power assay.

Chlorella samples showed a significantly higher ($p < 0.05$) Fe^{2+} chelation than all other trout samples. Yin et al. (186) showed *Chlorella sorokiniana* 3h hydrolyzed sample contained Fe^{2+} chelation activity that was very close to 100 ppm (~342 μM) EDTA activity. This shows *Chlorella sorokiniana* hydrolysate is capable of generating hydrolysates with high Fe^{2+} chelation activity.

Significantly higher Fe^{2+} chelation activity in *Chlorella sorokiniana* protein hydrolysates and molecular weight fractions indicate its ability to be used as Fe^{2+} chelating agents. Sou et al. (187) reported that the metal chelating activity of peptides might be related to the combined effects of the indole, benzene, and phenol rings existing in Trp, Phe, and Tyr and Trp and Phe. Therefore, the interaction of amino acids and the relative location in the sequence can significantly influence the antioxidant capacity of peptides.

4.3.3. Radical scavenging ability

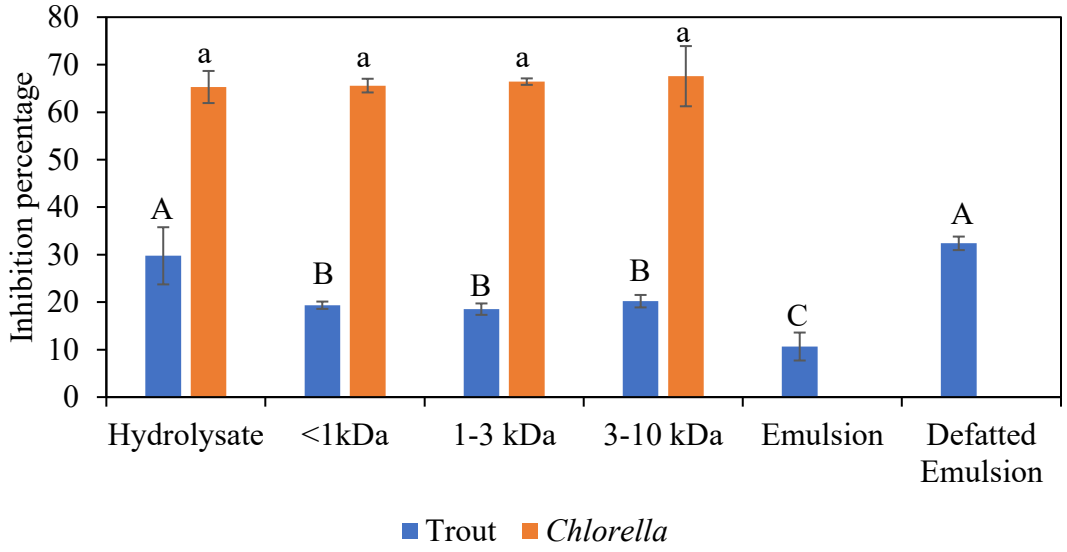

Figure 15: Radical scavenging ability of lyophilized trout, *Chlorella* hydrolysates, molecular weight fractions, and trout emulsion and defatted emulsion at 2mg/mL concentration. *Note:* Bars (mean ± standard deviation, n = 3) with different alphabets have mean values that are significantly different at $p < 0.05$.

DPPH is a stable nitrogen-centered free radical compound and is widely used to evaluate peptide, phenolic, and food antioxidant capacity (188). DPPH radical-scavenging activity indicates the hydrogen-donating ability of an antioxidant (181). The ability of trout by-product and *Chlorella sorokiniana* hydrolysates, separated molecular weight fractions and trout emulsion, and defatted emulsion fractions to scavenge DPPH radical is shown in Figure 15. The results clearly indicated that all *Chlorella* samples tested exhibited significantly higher ($p < 0.05$) antioxidant activity against DPPH than trout samples. There was no significant difference observed among the *Chlorella* samples ($p < 0.05$). Trout hydrolysate and defatted emulsion showed significantly higher radical scavenging activity than other trout MWCO fraction samples.

In previous literature studies, peptide fractions with lower molecular weights were found to have higher DPPH radical scavenging activity than their protein hydrolysate counterparts, which contrasts with results obtained in this study (178,189). In general, the radical scavenging activity of food protein hydrolysates depends on a variety of factors such as specificity of the proteases used in the protein hydrolysis, size, amino acid composition of the peptides, and the DPPH assay conditions (190). In addition, it is also reported that aromatic amino acids such as tryptophan and tyrosine impart strong antioxidant effects corresponding with the reported amino acid profile of the hydrolysates showing an abundance of hydrophobic amino acids, which may have influenced their DPPH radical scavenging activity (64).

A significantly higher DPPH radical scavenging suggests that *Chlorella sorokiniana* protein hydrolysates and their molecular weight fractions can be used as radical scavenging agents. This may be due to the higher occurrence of hydrophobic aliphatic (Val, Iso and Leu) and hydrophobic aromatic (Phe and Tyr) amino acid residues in the *Chlorella sorokiniana* protein hydrolysates and their molecular weight fractions (Figure 12 and Table 8) (105). Therefore, results indicate that *Chlorella* hydrolysates can be used as an auxiliary supplement to enhance the radical scavenging activity of trout bioactive peptides.

4.3.4. Angiotensin-converting enzyme inhibition

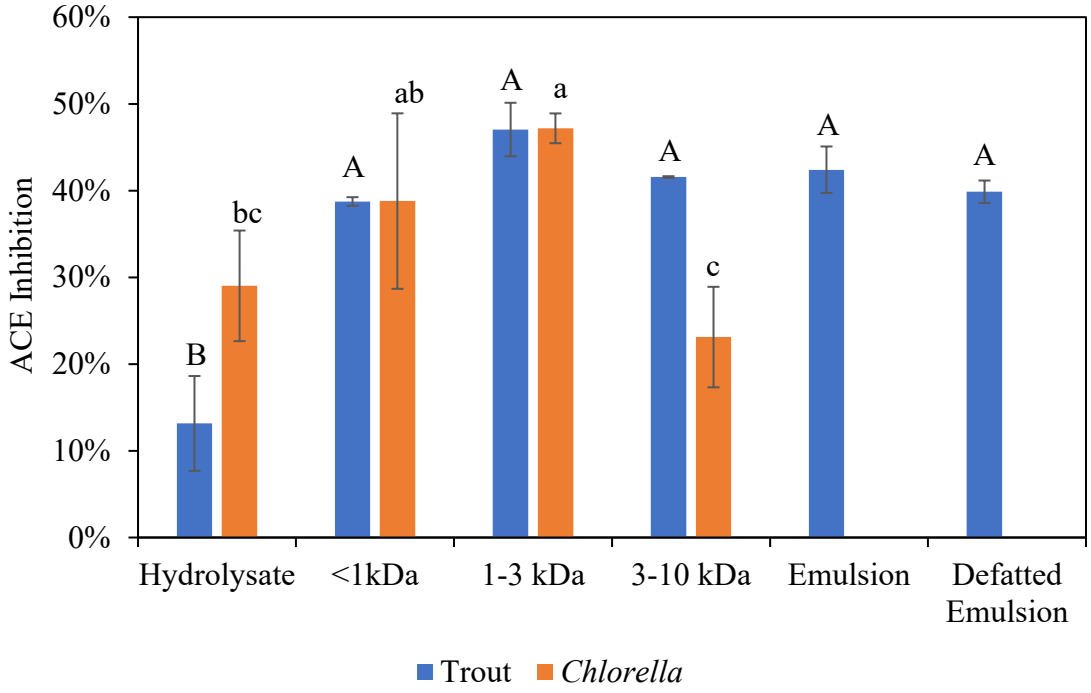

Figure 16: ACE inhibitory activity of lyophilized trout, *Chlorella* hydrolysates, molecular weight fractions, and trout emulsion and defatted emulsion at 2mg/mL concentration. *Note:* Bars (mean ± standard deviation, n = 3) with different alphabets have mean values that are significantly different at $p < 0.05$.

As shown in Figure 16, all the protein hydrolysates and peptide fractions exhibited ACE-inhibitory activities. The results demonstrated that there are ACE-inhibitory peptides present in hydrolysates of trout and *Chlorella sorokiniana* proteins. In comparing *Chlorella* and trout, both hydrolysates and their respective 3-10 kDa fractions showed a significant difference ($p < 0.05$). 1-3 kDa fractions of both trout and *Chlorella* showed the highest inhibition, followed by <1 kDa fractions. Trout complete hydrolysate exhibited a significantly lower ($p < 0.05$) ACE inhibition compared to other trout molecular weight fractions analyzed. Trout emulsion and defatted emulsion fractions demonstrated high

ACE inhibition (~40%) but were not significantly different. In *Chlorella,* 3-10 kDa fraction exhibited the lowest ACE inhibition among the *Chlorella* samples. In a previous study (178) with *Chlorella sorokiniana* <5kDa fractions showed the highest ACE-inhibitory activity (34.29% ± 3.45%), and crude hydrolysates demonstrated lower ACE inhibition activities. These results are in general agreement with results obtained in the current study. However, the inhibition activity recorded in this study was higher (~47 % inhibition) compared to that of the study done by Tejano et al., (178). The data also indicate that fractionation was effective in increasing the ACE-inhibitory activity of the hydrolysates. Pepsin hydrolysates from *C. vulgaris* protein waste (61), *C. vulgaris* biomass (63), and *S. platensis* biomass (63) were also reported to have high ACE-inhibitory activities. Moreover, several researches reported enzymes' effectiveness in producing hydrolysates and peptide fractions with high ACE-inhibitory activity (178,191). In literature, it was observed that hydrophobic amino acids are the most effective ACE inhibitors, especially those with tryptophan, phenylalanine, tyrosine and proline in the C-terminal and positively charged amino acids (arginine and lysine) and glycine, valine, leucine, and isoleucine in the N-terminal (144). In this study *Chlorella* hydrolysate contained higher amounts of arginine and was rich in glysine, alanine, proline leusine and isoleusine. Rainbow trout by-products hydrolysate contained higher glycine and was rich in alanine, leusine, lysine proline and valine. Amount of these amino acids in higher concentration might have increase their occurnece in the right places of the peptides to exert ACE inhibition activity.

Finally, according to the observed results for trout and *Chlorella*, 1-3 kDa fraction shows the highest ACE inhibition activity in vitro, and at that molecular weight, there was

no significant difference in ACE inhibition between trout and *Chlorella*. However, there could be synergistic enhancement or suppression associated with these two types of peptides due to the molecular level interactions.

4.3.5. Peptide Sequencing of 1-3 kDa fractions of hydrolysates

1-3 kDA fraction of both trout and *Chlorella* were selected for peptide sequencing to identify the peptides occurring in the most bioactive fractions. Peptide sequencing identified a total of 619 peptides from trout 1-3 kDa fraction matching with 86 proteins in the database for *Oncorhynchus mykiss* and 6587 peptides from *Chlorella* 1-3 kDa fraction matching with 572 proteins in the database for *Chlorella sorokiniana*. The most abundant 10 sequences observed in both trout and *Chlorella* 1-3 kDa fraction are listed in Table 5.

Table 5: Most abundant 10 amino acids sequences identified from trout and *Chlorella* 1-3 kDa fraction

#	**Trout (*Oncorhynchus mykiss*)**	**Chlorella (*Chlorella sorokiniana*)**
1	Gly-Gln-Asp-Gly-Leu-Ser-Phe-Pro-Gly-Pro-Pro-Gly-Pro-Pro-Gly	Val-Glu-Trp-Tyr-Gly-Pro-Asn-Arg-Pro
2	Gly-Gln-Asp-Gly-Leu-Ser-Phe-Pro-Gly-Pro-Pro-Gly-Pro-Pro	Val-Glu-Trp-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe
3	Gly-Pro-Ser-Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro-Pro-Gly	Arg-Glu-Ile-Glu-Val-Ile-His-Ala-Arg-Trp
4	Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro-Gln-Gly-Pro-Pro-Gly-Pro-Pro	Arg-Glu-Leu-Glu-Val-Ile-His-Ala-Arg-Trp
5	Gly-Pro-Ser-Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro-Pro	Ser-Val-Lys-Glu-Leu-Asp-Ala-Lys-Gly-Asn-Ala-Asp-Gly-Phe-Gly-Gly-

		Glu-Phe-Val-Val-Pro-Ser-Tyr-Arg-Gly
6	Pro-Ala-Gly-Pro-Pro-Gly-Asn-Pro-Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro	Ile-Pro-Ser-Lys-Trp-Asn-Pro-Ser-Lys-Glu-Gln-Asp-Phe-Pro-Gly-Val-Val-Leu
7	Gly-Pro-Ser-Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro	Ala-Arg-Asp-Leu-Asp-Leu-Pro-Gln-Gly-Val-Arg-Asp-Gly-Leu-Ser-Gln-Ala-Arg-Glu
8	Ala-Asn-Gly-Phe-Pro-Gly-Pro-Lys-Gly-Pro-Pro-Gly-Pro-Pro-Gly	Val-Glu-Phe-Tyr-Gly-Pro-Asp-Arg
9	Gly-Pro-Ala-Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro-Pro-Gly-Glu	Val-Glu-Phe-Tyr-Gly-Pro-Asp-Arg-Ala-Lys-Trp
10	Gly-Pro-Pro-Gly-Pro-Asn-Gly-Asn-Pro-Gly-His-Ala-Gly-Pro	Pro-Gly-Gly-Glu-Arg-Val-Pro-Phe-Leu-Phe

It is observed that the 10 most abundant sequences combined (shown in table 5) accounted for 50.55% and 12.92% of total peptide sequences for trout and *Chlorella sorokiniana,* respectively. The most abundant trout by-product hydrolysate peptide sequence (Gly-Gln-Asp-Gly-Leu-Ser-Phe-Pro-Gly-Pro-Pro-Gly-Pro-Pro-Gly) accounted for 13.10%, while the most abundant peptide sequence (Val-Glu-Trp-Tyr-Gly-Pro-Asn-Arg-Pro) in *Chlorella spp,* accounted for only 3.38%. Sequences observed for trout 1-3 kDa fraction indicate the amino acid pattern of Gly-X-Y, which is identical for collagen proteins (192), indicating the most abundant peptides derived from trout collagen proteins.

Kim & Byun (51) identified Lys-Val-Asn-Gly-Pro-Ala-Met-Ser-Pro-Asn-Ala-Asn as an ACE inhibitory peptide from enzymatic hydrolysates of rainbow trout (Oncorhynchus mykiss) muscle. However, this sequence was not detected in the current study. Previous studies on the relationship of peptide sequence and ACE inhibition reported that peptides with proline or an aromatic residue (Phe, Trp, Tyr) at their C-terminal positions and a branched aliphatic (Val, Ile, or Leu) amino acid residue at N-terminals showed higher ACE-inhibitory activity (192,193). Trout by-product hydrolysate peptide sequences contained had an abundance of proline and hydrophobic amino acids such as Phe, and Leu which may have increased the ACE inhibition in 1-3kDa fraction.

Although a range of previously reported short sequence peptides (Table 2) have been reported in the literature, isolated from *Chlorella* spp, no previously reported long chain peptides were identified and no comparative data for both trout and *Chlorella* 1-3 kDa fractions. Nevertheless, dipeptide sequences (Trp-Val, Val-Trp, Ile-Trp, and Leu-Trp) reported as having ACE-inhibitory activity by Lin et al., (66) were detected within the observed sequences of *Chlorella.* Thus, these dipeptide sequence occurrences may have contributed to the ACE inhibition activity in the *Chlorella* 1-3 kDa fraction.

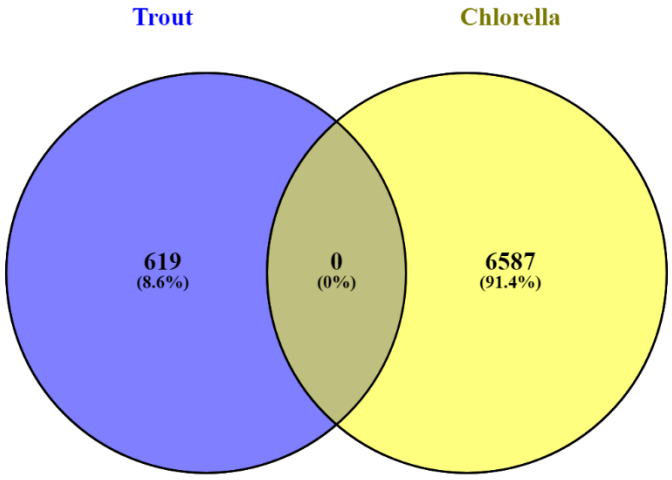

Figure 17: Peptide sequence analysis for similarity of trout and *Chlorella* 1-3 kDa fractions

It is also observed that there was no overlap of similar peptide sequences detected from both sources in 1-3 kDa fraction (Figure 17). As these peptides originate from two different species that are not closely related evolutionary, they are expected to have no overlapping peptide sequences (non-homologous proteins). But even though these hydrolysate fractions are not similar in amino acid sequences, they have shown similar bioactivities such as ACE inhibition and antioxidant properties, which suggest that the mechanism of action of these two-peptide fractions could differ from each other or the variety of peptide end groups with a similar mechanism of action. Therefore, as expected, there is the capacity to use these peptides fractions as complementary sources to boost bioactivities with complementary characteristics of marine origin to serve the need for a range of functional ingredients in the food that can provide a variety of health benefits.

GRAVY (grand average of hydropathy) is used to represent the hydrophobicity value of a peptide, which calculates the sum of the hydropathy values of all the amino acids

divided by the sequence length (194). GRAVY value for the most occurring 50 sequences resulted in an average of -0.9690 and -0.3018 for trout by-product mix and *Chlorella sorokiniana,* respectively. This indicates that *Chlorella* amino acid sequences are more hydrophobic than trout amino acids. Since hydrophobic amino acids were previously reported to increase the antioxidant properties (195). This increase in hydrophobicity may have resulted in higher antioxidant capacities observed in *Chlorella* fractions than trout fractions throughout this study. In amino acid sequences of *Chlorella* 1-3kDa fraction, occurrences of hydrophobic amino acids such as alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), and phenylalanine (Phe) also indicate the higher hydrophobicity than trout 1-3kDa fraction amino acid sequence which is also confirmed by the amino acid composition (figure 12).

CHAPTER 5: CONCLUSION

The overall goal of this study was to compare peptides and hydrolysates generated from Rainbow Trout (*Oncorhynchus mykiss*) by-products and Microalgae (*Chlorella sorokiniana*), for their functional properties in order to compare and assess their different attributes and how these compare and complement for use in food or supplements. Marine by-products generated from fish, crustaceans, and algae (micro and macro spp) have gained the attention of researchers for the production of highly functional peptides. Though the fish by-products have been demonstrated to be a protein-rich sustainable resource for the generation of bioactive peptides, there may be supply variations and bioactivity deficiencies. Many plant sources have been explored for functional ingredients, particularly plant and algal proteins. This study is the first to compare and characterize peptides from microalgae with those of marine sources in order to understand opportunities for targeted use or synergies in the application as antioxidants and antihypertensive use. Supplementing complementary sources of peptides to generate products with higher or complementary bioactivities may present novel products for use in supplements or functional food applications. This research has elucidated key differences in activity related directly to differences in peptide variation, frequency, and amino acid sequence. This data provides a valuable side-by-side comparison of marine and algal peptides for these two applications and reveals the following.

1. Peptides generated from microalgae (*Chlorella sorokiniana*) in the 1-3kDa fraction are unique in amino acid composition and sequence and activity from peptides generated in fish (*Oncorhynchus mykiss*) 1-3 kDa, as a result of the differing proteinaceous parent plant and marine source materials.

2. Microalgae (*Chlorella sorokiniana*) processed using a combined microwave and enzyme treatment method produced highly functional bioactive peptides, despite the relatively low yield.

3. In all evaluated antioxidant assays (FRAP, Fe^{2+} chelating ability, and radical scavenging ability), *Chlorella sorokiniana* has shown significantly higher activity than the trout by-product peptides despite the lower protein concentration in the hydrolysate.

4. In the current research, the ACE inhibitory activity exhibited by both hydrolysates irrespective of molecular weight fractionation was similar. Therefore, both trout and *Chlorella sorokiniana* can produce equally potent ACE inhibitory peptides using differing methods of hydrolysis and comprising very different peptide sequencing.

Chlorella sorokiniana biomass has produced bioactive peptides with a unique profile and with specifically higher antioxidant bioactivity than Trout bioactive peptides. Thus, plant-based micro-algal peptides can be utilized to help not only to overcome the supply limitations of marine biomass by having a comparable high-value peptide stream but to biologically manufacture peptides with similar or greater bioactivity, which can serve the need for a range of functional ingredients in food to provide a variety of health benefits.

This also presents an opportunity to use algal peptides to supplement marine resources for other applicationss, creating increased potential for upcycling fish by-products. Furthermore, results have shown supplementing these two sources will increase the complexity of the resulting combinations of hydrolysate and could increase or exhibit synergistic effects in different types of antioxidant activities and ACE inhibition. Further

research is required for the isolation of these abundant amino acids and evaluating differences in bioactivities. Further development and optimization of extraction processes for microalgal peptides are required to enhance yield from this highly valuable resource that can be produced cost-effectively and consistently. Combining the two hydrolysates might result in synergistic products. Still, it would require evaluation of in vivo effects to truly determine whether increasing antioxidant combinations that target multiple pathways is safe and beneficial for preventative or therapeutic health.

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