BIOPROCESSING OF ATLANTIC SALMON (*Salmo salar*) BY-PRODUCTS: RECOVERY AND ANTIOXIDANT PROPERTIES OF PROTEIN HYDROLYSATES

by

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Submitted in partial fulfillment of the

requirements for the degree of Master of Science

at

Dalhousie University

Halifax, Nova Scotia

May 2017

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ABSTRACT

Yield, functional properties and antioxidant activities of salmon by-product protein hydrolysates derived from processing waste were investigated. Atlantic salmon by-products were ground and bioprocessed with the addition of formic acid/ lactic acid bacteria/ Flavourzyme to recover protein hydrolysates with varying functionalities. Formic acid treatment and lactic acid fermentation were facilitated by endogenous proteases (autolysis) in the salmon tissues and resulted in higher hydrolysate recovery from longer processing duration. While the autolytic hydrolysate fractions demonstrated higher sacrificial antioxidant properties, Flavourzyme derived hydrolysates were found to have higher Fe(III) reduction capacity and Fe(II) chelation capacity. Protective antioxidant mechanisms were observed for salmon by-product protein hydrolysate treatment within the plasma matrix and HT29 cellular model subjected to oxidative stress.

LIST OF ABBREVIATIONS AND SYMBOLS USED

Terms/abbreviations	Definitions
ANS	1-anilinonaphthalene-8-sulphonate is an anionic fluorescent dye used in protein surface hydrophobicity determination
Autolysis	Degradation or proteolysis induced by inherent enzymes in the tissue
DH	Degree of hydrolysis, is calculated to measure the extent of proteolysis
DMSO	Dimethyl sulfoxide
DPPP	diphenyl-1-Pyrenylphosphine, is used in fluorescent based detection of lipid peroxides. DPPP is essentially nonfluorescent until oxidized to a phosphine oxide by peroxides
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid) (Ellman's Reagent), used in colorimetric determination of sulfhydryl groups
E/S ratio	Enzyme to substrate ratio
EDTA	Ethylenediaminetetraacetic acid, a chelating agent (used as positive control)
FA	Formic acid treatment
FAN	Free amino nitrogen content
Fe(II) chelation	Ability or capacity to chelate Fe^{2+} ions from solution
Fish by-products/processing waste	Wastes or unused components generated from marine processing industries, which primarily consists of heads, fins, skin, frame and viscera
FL	Flavourzyme proteolysis
FRAP	Ferric reducing antioxidant potential is a measure of the capacity (antioxidant power) of certain substances to reduce the Fe^{3+} ions to Fe^{2+} ions in solution
GSH	Glutathione, a naturally occurring reducing agent in cells
GSH protection	A measure of the capacity of substances to protect glutathione from Fenton's oxidation induced by Fe^{2+} and H_2O_2
H-MW	Higher molecular weight
HT-29	Human colon adenocarcinoma cell line that expresses characteristics of

	mature intestinal cells
L-MW	Lower molecular weight
LA	Lactic acid fermentation
Liquefaction	Degradation of structural components of ground tissues resulting in release of inherent moisture from the mix
mBBr	Monobromobimane, used in fluorescence based detection of glutathione
MRPs/iMRPs	Maillard reaction products/ intermediate Maillard reaction products
Protein recovery	Indicates the quantitative levels of protein assimilated through a processing approach
Residual fraction	The lowermost layer largely composed of bone and other heavier and insoluble sediments, formed following the centrifugation of processed fish tissues
ROS	Reactive oxygen species
SH	Sulfhydryl or thiol (group or content)
Sludge fraction	Sandwiched between oil and aqueous layer, this layer is largely composed of emulsified oil and fat along with other lower density materials that can interact with oil, formed following the centrifugation of processed fish tissues
S_o	Surface hydrophobicity
Soluble protein content	Protein content as determined by modified Lowry assay
Soluble/aqueous fraction	The aqueous layer which has the largest extent of moisture, formed following the centrifugation of processed fish tissues
Total soluble protein recovery	The total soluble protein recovery establishes the net mass of soluble protein extracted per 100 g of ground fish tissues
ρ	Spearman's rank order coefficient

ACKNOWLEDGMENT

I express my sincere gratitude to both my co-supervisors, Dr. Beth Mason and Dr. Chibuike Udenigwe for both their continuous support, guidance, encouragement and mentorship throughout the program. I appreciate and thank the contributions in the form of comments, suggestions and feedback of my committee members, Dr. Andrew Swanson and Dr. Stephanie Collins. I am deeply indebted to Dr. Zied Khiari, Andrew Crowell, and Elizabeth MacCormick for their guidance, assistance in conducting various experiments. I also thank the administrative and financial support from Dalhousie University, Verschuren Centre (Cape Breton University), NSIRT, Mitacs, Saputo and AOCS.

I would like to acknowledge and extend my gratitude to Dr. Shine (Xu) Zhang, Wei Xu, Chigozie Okolie, for providing their help in various forms for the completion of this project. I am thankful to Anne LeLacheur, Debi Walker, Daryl Mullin, Marie Law, Pamela Sutherland, Diane Patterson, Haibou Niu and Stephen Kelloway for their help. I appreciate the contributions of graduate student association colleagues Carolyn Marshal, Hannah Mawhinney, Lindsay Gillard Carolyn Mann, Carolyn Wilson, William Hannah.

My heartfelt thanks to Prabahar Ravichandran, Janvi, Parghat Gopal, Gayathri Ilangumaran, Akash Kulshreshtha, Palaniappan Ramasamy, Rakesh Menapati, Deney Joseph, Jennifer Klaus, Fenzang Wang, Yuanyuan Jiang, Dimple Kaushal for their help and support. Many thanks to all my friends in India KK, Kannan, Nijil, Pradeesh, Surjith, Sujith, Hassan, Yadu and LD for checking up on me from time to time.

Amma and achan, you are the best parents anyone could hope for, THANK YOU for putting up with me all these years!

Aishwarya, for always being there

CHAPTER 1

INTRODUCTION

Fish and other aquatic products being rapidly perishable, have a significant requirement for post-harvest processing. A large proportion of the inputs that goes into these industries are turned into by-product waste. Therefore, fish processing waste streams are widespread and in abundance. Atlantic Canada especially, is heavily dependent on fisheries and generates large amounts of marine by-products. According to a report by Statistics Canada, most of the permits issued by Environment Canada for solid waste disposal at sea, was for fish waste in the Atlantic region (2010-11).[1] Similarly, counties such as Australia spend around $15 million annually for discarding processing waste from the seafood industry.[2] In contrast, countries in Europe have several regulations in place to minimize the impact of fish processing wastes on the environment.[3] Disposal of fish processing waste into the environment has a heavy toll, since high amounts of organic matter present in these wastes, influence changes to the assemblage of community structure and native biodiversity, especially in benthic ecosystems.[3,4]

Owens and Mendoza have extensively reviewed processing approaches (enzymatic hydrolysis, autolysis and bacterial fermentation) used to prepare a wide range of products from fish by-products.[5] Even though commercial-enzymatic approaches are expensive (high cost of enzymes), this approach is being extensively employed in research and development of fish protein hydrolysate based products.[6,7] Autolytic methods (utilizing endogenous enzymes in fish tissues), on the other hand, are cheaper and sustainable when compared to commercial proteases. Microbial (fermentation) and autolytic processing however, are considered to be complex processes with relatively low capability of designing protein hydrolysates with specific functionalities.[8] Another level of complexity to bioprocessing with autolytic approaches involves

the variability in endogenous enzymes within fish tissues, which changes with species, season, age, gender, maturity, diet and other environmental factors.[8]

A wide range of products have been derived from fish by-products through research endeavors directed at the utilization of fish wastes. Despite the high content of food-grade proteins, these resources are mostly underutilized as raw materials in the production of fishmeal, fertilizer and animal-feed. Fish silage is a liquid product formed from the liquefaction of fish tissues and is used in poultry and animal-feed. Ensilation is an autolytic process carried out by endogenous enzymes present in the fish tissue and is accelerated *via* addition of acid, inducing optimum conditions for enzyme activation facilitating the breakdown of tissues and limiting the growth of spoilage bacteria.[9] Fish surimi is prepared from mechanically deboned, minced fish meat that is washed with water and often treated with cryoprotectants (such as protein hydrolysates) to conserve gel-forming ability.[10] Fish sauce preparation in Southeast Asian countries has been traditionally followed with fish muscle/by-products, utilizing microbial fermentation in a high salt concentrated mixture of blended tissues, resulting in a sluggish process of protein hydrolysis.[11] More recently, several reports have found applications for these by-products in biodiesel production,[11] food packaging (chitosan),[12] cosmetics (collagen),[13] food supplements (omega-fatty acids),[14] moisture and texture management in foods (protein hydrolysate),[15] salad dressing (protein hydrolysate),[16] functional biomolecules (bioactive peptides,[17] carotenoids,[14] glycosaminoglycans)[18], protein bars[19] and protein shakes.[3]

Biologically active peptides/protein hydrolysates can play important roles in metabolic regulation within living systems, in addition to being a source for nutrition. It is well established that emergence of pathological conditions (diseases, inflammation etc.) often result in oxidative imbalance/stress that leads to generation of free radicals. Oxidative damage and lipid

peroxidation is also a major issue associated with food processing and storage. Peptides derived from natural sources such as fish products have been extensively reported to exert antioxidant activity that mitigates oxidative stress *via* chelation of pro-oxidant metals, quenching of free radicals, inhibition of lipid peroxides and several other mechanisms, thereby providing beneficial effects to human health as well as food processing.[20] Several sources of proteins are being explored for the production of biologically active peptides. Utilizing by-product streams such as whey, oilseed meal, fish processing waste are especially attractive as these are cheaper and rich sources of protein. And for reasons mentioned earlier, utilizing marine bioprocessing waste for the production food-grade protein hydrolysates is promising in Atlantic Canada. Some of the approaches that have been actively pursued for generation of protein hydrolysates or bioactive peptides from marine processing waste which has been reviewed in Chapter 2.

CHAPTER 2

LITERATURE REVIEW

Bioprocessing approaches for fish by-product proteolysis: A perspective on yield, functionality and commercial preparation

Abstract

Protein hydrolysates have been developed and evaluated for several health-benefit applications in the past decade. Utilization of marine by-products, a major source of food waste, to generate functional foods and nutraceuticals has benefits to health as well as to the environment. Chemical, microbial and enzymatic approaches have been employed for protein hydrolysate preparation, with the high cost of the enzymes and lack of substantial evidence for health claims, limiting industrial applications. However, a number of diverse physiochemical activities have been attributed to by-product derived protein hydrolysates in recent times. This review presents the different approaches that are employed to recover protein hydrolysates from marine industrial by-products from the perspective of utilizing food-grade proteins available in these resources for functional foods.

2.1. Introduction

Around 60% of the input used in marine processing industries is generated as by-product waste, which consists of heads, fins, skin, frame and viscera.[21] Although they are used in fish oil production, the edible protein components in these processing by-products remain underutilized. These are currently being processed mostly into lower value commodities such as fish meal, animal feed and fertilizer. Therefore, food researchers around the world are looking at possible ways to utilize these rich sources of protein. Utilization of these by-products will have economic and environmental beneficial effects, enhancing the sustainable use of marine resources.

Food components play a significant role as health-benefiting factors to help alleviate disease conditions and stress. These have gained much interest, particularly in light of the extensive availability of large quantities of processing wastes. However, in spite of extensive research and development, very few high-value products have been commercialized from marine waste streams.[22] Cost of isolation of specific functional components remain one of the most challenging aspects for establishing products capable of getting to the market.[22]

Processing of fish products has been in use since the medieval times. A great diversity of traditional preparations, especially preserved fishery products involves enzymatic hydrolysis, bacterial/fungal fermentation or chemical (salt/acid) treatment.[5] It is well established that the level of protein recovery correlates positively with the degree of hydrolysis (DH).[23] As the protein is broken into smaller pieces, the solvent accessibility increases, thereby increasing the soluble protein content in the form of peptides. Enzyme technology in protein recovery has been forecasted to be necessary in the production of a wide range of food ingredients and industrial products.[8] Enzyme activity is governable and can be tailored for desirable molecular properties and functionalities of hydrolysates. Several studies have demonstrated marine sources of proteins

to release peptides on proteolysis, which function as potential antioxidative agents.[24–27] However, industrial applications have been severely limited by cost of protease preparation and application on a commercial scale.

This review focuses on the different approaches used to generate protein hydrolysates from marine by-products. The effectiveness of individual approaches is discussed considering parameters such as crude protein/peptide recovery and functionality of the hydrolysates.

2.2. By-product protein concentrate preparation

Characterization and yields of by-products has revealed variance in the amounts and protein product for the same species harvested and processed at different locations. For example, Atlantic salmon (*Salmo salar*) by-product yields from Chile[21] when compared to those caught and processed in South Australia[2] demonstrated major differences. Marine by-products such as fish skin, bones, heads, viscera and residual minced meat are relatively cheap and their utilization in the production of bioactive peptides will result in a reduced food processing cost, with an added advantage of value addition to waste. However, extraction/isolation of proteins on a large scale from these waste products is an expensive process and is not economically feasible. Several studies rely on chromatographic approaches for the separation of proteins or peptides following processing (Reviewed by Kitts and Wheeler, 2003).[28] Chromatographic approaches however, are not scalable to commercial applications. Additionally, use of extended duration processing techniques for protein concentrate preparation is associated with protein degradation products and loss in nutritional quality, which is not ideal.

Currently, membrane technology is being employed for large scale protein concentrate preparation, especially in the dairy industry, and even for marine sources.[29,30] Application of nano/ultrafiltration approaches has been extensively reviewed for recovery of proteins from

seafood processing waters.[31] Bioreactor coupled to an ultrafiltration unit has been demonstrated to facilitate protein recovery from Sardine waste in pilot scale (60 L) utilizing crude peptic enzyme extracts.[29] However, Benhabiles et al. (2013) demonstrated that irreversible membrane fouling occurs during recovery which may limit usefulness of the filtration approach unless effective membrane cleaning methods are applied.[30]

2.3. Production of marine by-product protein hydrolysate based products

A wide spectrum of marine by-products have been utilized for the production of protein hydrolysates (extensively reviewed by Halim et al. 2016).[32] Additionally, the more conventional industrial uses of marine by-product are directed towards ensiling for animal-feed and fishmeal applications.[8] However, considering the quality and abundance of edible proteins in these materials, their application in fishmeal, fertilizer preparation, and even animal-feed, is underutilization of these resources and much more valuable outcomes are possible from using this resource.

A plethora of enzymatic, biological and chemical methods have been employed for the production of bioactive peptides. In contrast, a few studies have also reported the antioxidant properties of fish by-product derived native peptides which are not subjected to any form of processing.[33,34] A higher temperature (60 °C) during processing facilitates denaturation of structural (collagen) and sarcoplasmic proteins (myoglobin) in fish tissues, thereby enabling increased hydrolysis.[35–37] However, higher temperature may also impact the activity of endogenous proteases and result in chemical modifications[38] as well as chemical interactions such as the formation of Maillard Reaction Products and lipid peroxides in a complex system.[39] The process of hydrolysis influences physicochemical properties such as molecular size, hydrophobicity and polar groups of the protein hydrolysates.[8] Protein hydrolysates prepared with

a higher DH have been shown to demonstrate higher solubility[40] and antioxidant activity.[27] The physicochemical properties and potential physiological bioactivities have significant impact with regards to health of humans and animals, if introduced in food or utilized as feed, respectively. By-products from filleting plants and by-catch are suitable for human consumption, however, proper food-grade handling of these protein sources is crucial.

Several studies that generate protein hydrolysate from marine by-products, report the addition of distilled water during the processing stage (preparation of protein concentrate[7,27,41–43] or by-product slurry[44–50]) or even during the isolation (generally by centrifugation) of hydrolyzed peptides. However, addition of water during the processing and its removal by freeze/spray drying is responsible for additional costs during processing and has been considered as a major barrier for the commercialization of protein hydrolysate product.[8] In addition, drying methods have been shown to influence proteinaceous functional properties, with lyophilization (freeze-drying) contributing to the highest quality attributes.[51]

2.4. Acid hydrolysis

Protein hydrolysis with strong chemicals, extreme temperatures and pH generally yields products with reduced nutritional qualities, altered chemistry, poor functionality, and restricted use in functional foods. Acid hydrolysis has major limitations regarding control and reproducibility and leads to destruction of pH-sensitive amino acids,[11] cross-linkages, chemical-modifications,[52] or isomerization (racemization).[53] Acid hydrolysis is especially used in treating shell material of crustaceans (shrimp, lobster, crab), wherein acid treatment fulfills the dual objective of hydrolyzing the proteins as well as demineralization of complex inorganic salts.

2.4.1. Recovery during processing and efficiency

In protein extraction from fish, acid treatment has been demonstrated to be more efficient as compared to alkali treatment.[54] Alkalis can induce a greater extent of disulfide linkage occurrence (pKa of S-H is 8.3, higher pH induces deprotonation of SH and in turn oxidation to disulfide bonds) in proteins, that leads to the formation of insoluble protein aggregates.[54]

Acid hydrolyzed marine wastes can be utilized as nutrient source for microbial fermentation, as can be seen in *Lactobacillus rhamnosus* fermentation, which showed improved production of lactic acid with acid hydrolyzed processing waste acting as a source of nutrients (as a substitute for yeast extract), in comparison to intact proteins.[55] Acid hydrolysis is effective in recovery of protein even from tougher protein sources. For instance, HCl induced hydrolysis on shrimp shell wastes yielded a protein content of 23.5%.[56] The study also demonstrated that the protein extraction time did not significantly increase the quantity of solubilized protein from shrimp wastes indicating that the processing is relatively fast and spontaneous.

2.4.2 Functionality

After hydrolysis, the acidic mixture of protein hydrolysate would have to be neutralized, which can lead to large amounts of salt accumulation, which needs to be further processed. The chemical residues from processing can impact overall compatibility, palatability and can interfere with food/feed matrices. Acid hydrolysis can be widely utilized to convert underutilized and secondary raw material from fish into fertilizer due to the low production cost and resulting extensive hydrolysis. However, use of strong mineral acid hydrolysis for feed/food application is a long way from possible because of the lack of use of food/feed grade materials during processing, modified functionality and uncontrolled nature of the entire process.

2.5 Chemical-induced Autolysis

Autolysis is a process by virtue of which, proteases inherent in by-product tissues/ground slurry are activated as a result of optimal conditions (pH and temperature). Generally, the approach involves the addition of a chemical (acid or base) to adjust the pH inducing the activation of endogenous enzymes, resulting in proteolysis. Formic acid treatment is a conventional and popular approach, used in ensilation of marine by-products for animal/poultry feed preparation.[57] A US patent was awarded to a process of recovery of pepsin and functional protein hydrolysate from cod viscera hydrolyzed in the presence of formic acid. Following hydrolysis, the processed viscera was subjected to solids and lipid removal, and ultrafiltered to recover protein hydrolysate and functionally active pepsin.[58] A formic acid treatment based approach is utilized in preparing H-pro® (Hordafor, Størving, Denmark), a commercialized proteinaceous product for animal feed, prepared from by-products of farmed salmon. Besides the activation of endogenous enzymes, formic acid (2% v/v) treatment of proteinaceous materials can also induce chemical cleavage of proteins at the aspartyl residues,[59] and this property is currently being utilized in protein identification (proteomics).[59]

In contrast, studies have also used plain pH adjustment (pH ~ 5.0 - 7.0 with NaOH) to induce the activity of endogenous enzymes within fish by-products.[48–50] Several previous studies have demonstrated a higher pH to be more efficient at proteolysis through endogenous enzymes (higher DH).[50,60,61] However, lower pH stabilizes the hydrolyzed tissue material and prevents the growth of harmful microbes.

2.5.1 Recovery during processing and efficiency

Autolysis of white shrimp head by gradual increase in temperature (5°C every 30s) resulted in the maximum DH (48.6%) and protein recovery of 87.4%, with an overall increase in nutritional quality.[37] Generally, the protein recovery from formic acid induced autolysis has been

reported to be around 80%.[9] Higher temperatures of incubation and higher degree of muscular parasitism was demonstrated to increase the autolysis of Pacific whiting (*Merluccius productus*) and this technique is considered to aid in fish protein hydrolysate production.[36] Autolytic activity has been demonstrated to be maximum at 60°C in several studies.[35–37] It would be expected that maximum enzyme activity to be optimal at physiological temperature, however, the higher DH and the corresponding maximum recovery are at a temperature of around 60°C, which is considerably higher than the physiological condition.

2.5.2 Functionality

Processing conditions have a very significant role in modulating the peptide profile and functionality of by-product hydrolysates. These conditions favor or select to a degree, activity of specific endogenous proteases in the complex system. Atlantic salmon (*Salmo salar*) muscle protein hydrolysates prepared with endogenous visceral proteases in salmon had different peptide profiles even at the same DH, when processing was carried out under different reaction temperatures.[62] Protein hydrolysate prepared with endogenous enzymes can be as effective as hydrolysates prepared with exogenously added enzymes. Atlantic salmon visceral protein hydrolysates at 5% and 10% inclusion levels (in diet) was demonstrated to increase broiler chicken growth performance when included in starter diets compared with either a plant protein-based or a fish meal diet.[63] Additionally, it can also be inferred from the above-mentioned study that the performance of autolytic hydrolysates in improving growth performance of broilers to be just as efficient as visceral protein hydrolysate prepared with the addition of papain and bromelain. In addition to production of hydrolysates, HCl assisted autolysis has also been utilized for the preparation and activation of functional crude enzymes from by-products.[29] These enzyme extracts can then be utilized in a better controlled proteolysis processing.[29]

2.6 Microbial fermentation

Traditional fish sauce production in Southeast Asia has always employed slow bacterial fermentation with high salt concentration over prolonged periods of time.[64] *Lactobacillus sp.* are commonly utilized as a means of preservation of food stuffs *via* fermentation. Lactic acid fermentation results in the production of lactic and acetic acid which activate endogenous proteases (Cathepsins) present in by-product tissues through the reduction in pH similar to the formic acid induced autolysis.[65] Reduced pH along with the antimicrobial chemicals (bacteriocins) produced by Lactobacilli inhibit the growth of other harmful bacteria. Studies have demonstrated fish by-product subjected to LA-fermentation substantially reduced the populations of harmful microbes (*Staphylococcus sp*., *Clostridium sp*. and coliform bacteria) as compared to raw offal from processing.[65,66] This wide application of this method across Asia is evidence of the potential for its use in other areas for human consumption in products developed using this processing approach for health and wellness. It is noteworthy to mention that the results obtained for non-sterilized media fermentations with *Lactobacillus casei* CECT 4043 were similar to those obtained for sterilized media, and pH stable fermentate was obtained following 72 hours.[67] It remains to be assessed whether microbial fermentation without pre-sterilization is applicable within the framework of Good Manufacturing Practice (GMP) and Hazard Analysis and Critical Control Point (HACCP) in production.[22] However, there are several traditional Asian food-products available currently, that utilize bacterial fermentation of unsterilized protein-based substrates. While homofermentative-microbes enable higher lactic acid production and lower pH-stabilized protein hydrolysates, heterofermentation is more suitable for production of flavor compounds associated with fish sauce production.[68]

Microbes also act as a source of proteases, they are advantageous over commercial enzymatic preparations, in that microbes are inexpensive to use and fast to cultivate/propagate. In addition, the microbes are enabled with a plethora of proteases (exogenously secreted or released as cells die (depending on the strain)). Besides different LAB species, other microbes such as *Aspergillus oryzae*[69] and *B. subtilis*[43] have also been used for fermenting fish by-products. The strain of microbes used for the hydrolysis can be selected based on their proteolytic potential,[70] as well as consumptability, for instance, the *Aspergillus* strains used in fermentations would need to be devoid of aflatoxin production. Based on the proteolytic machinery, peptides with desired properties, different amino acid sequences and free amino acids may be formed. The degree of proteolysis, cleavage pattern, and change in pH are factors that are highly dependent on the microbial species involved in fermentation and the physicochemical conditions during the course of processing. Peptides and amino acids released from proteins during fermentation along with flavor compounds, often underlie the functional, rheological, sensory and biological properties of the fermented product.[53]

2.6.1 Recovery during processing and efficiency

Recovery of protein hydrolysate is influenced by tissue type and other processing parameters such as type of inoculum, amount of inoculum, processing duration, temperature, pH and stirring/shaking conditions. The broad range in protein yields from by-products is dependent on the bioprocessing approach as well as the properties of substrate.

Microbes isolated from earthworm viscera has been demonstrated to convert fish waste into liquid fertilizer with 5.71% protein content.[71] Whereas, lactic acid fermentation of shrimp wastes yielded peptide content of up to 46% in the hydrolysates.[72] Fermented fish meat protein hydrolysates prepared from Sardinella, zebra blenny (*Istiblennius zebra*), goby and ray with

Bacillus subtilis A26 achieved a high recovery of up to 81% protein content (30% DH) in the hydrolysates.[43] Contrastingly, cod protein hydrolysate with 58% protein yield (45% DH) was prepared with *Bacillus sp*.[73] Interestingly, despite a lower DH, the recovery in the case of fish meat hydrolysates was higher as compared to by-product cod hydrolysate indicating that recovery depends also on source tissue type and initial protein content.[73] It is not always possible to compare protein recovery of different studies, in terms of the effect of DH on it, since different studies use different approaches to calculate both protein recovery and DH.

2.6.2 Functionality

Biological treatments have been shown to be more compatible, especially with regards to chemical modifications,[52] peroxide levels,[74] and stability of amino acids[11] as compared to chemical treatments. *Acanthogobius hasta* by-product subjected to solid state fermentation with *Aspergillus oryzae* enhanced radical scavenging activity, reducing capacity and metal chelation capacity of the hydrolysate.[69] Similarly, fermentatively (LAB; *Pediococcus acidilactici* NCIM5368, *Enterococcus faecium* NCIM5335 and *Pediococcus acidilactici* FD3) recovered protein hydrolysate from Rohu (*Labeo rohita*) and Catla (*Catla catla*) heads demonstrated good radical scavenging activity and antibacterial activity.[75] In addition, another study comparing acid and fermentative hydrolysis (*Enterococcus faecium* NCIM5335) found fermentative-hydrolysate to exhibit higher radical (hydroxyl, ABTS, DPPH) scavenging activity and antagonistic activity against *Salmonella typhi* FB231.[76] Whereas, the comparison of antioxidant and antibacterial activity of fermentative (*Pediococcus acidilactici* NCIM5368) protein hydrolysate with enzymatic (Alcalase) hydrolysate revealed similar radical scavenging activity and higher antibacterial activity (of fermentative hydrolysate) against *Listeria monocytogenes*.[77] As with

other processing approaches, emulsifying and foaming properties of peptides are governed by the size of peptides and the concentration employed.

2.7. Addition of proteolytic enzymes

Proteolytic enzyme preparations used in food applications can be broadly categorized as narrow cleavage-specific and broad cleavage-specific enzymes based on the peptide bond hydrolysis pattern. Most of the digestive enzymes along with plant-derived proteases such as bromelain and papain are narrow-specific. Whereas, the enzymes secreted by or derived from microbial cultures are broad-specific.

2.7.1. Biological extracts and digestive enzymes

Biological extracts of enzymes are also derived from by-products and are hence cheaper alternatives to purified enzymes. Digestive enzymes and enzymatic extracts consist mostly of analogs of pepsin, trypsin and chymotrypsin. These proteases have narrow cleavage-specificity as compared to microbial protease preparations.

Evaluation of bioactive peptide production using simulated gastrointestinal conditions (digestive enzymes) is also sometimes carried out with the objective of ensuring stability of peptides. Peptides generated with this approach are thus, most likely to survive conditions in the gastrointestinal tract when consumed. Multifunctional sea cucumber hydrolysates have been demonstrated to be resistant to simulated gastrointestinal digestion.[78]

2.7.1.1 Recovery during processing and efficiency

Because of the narrow specificity, digestive enzymes could be expected to have relatively low DH. However, in contrast to the prediction, Atlantic salmon pyloric caeca extract was demonstrated to have comparable DH as commercial alkaline proteases (Alcalase, Flavourzyme, Corolase).[47]Another report comparing several enzymes (Alcalase, Flavourzyme, Neutrase,

pepsin, Protamex, and trypsin) found protein recovery to have not varied, remaining around 10% for salmon pectoral fin with crude protein content of 17.2%.[79] Additionally, concentration of viscera extract (influencing E/S (Enzyme/substrate) ratio), reaction duration and DH were demonstrated to be closely correlation with the nitrogen recovery from the ponyfish (*Gazza minuta*) muscle.[80] Higher DH has also been implicated in better protein recovery from blue shark skin[81] and other marine by-products.[82,83] Crude pepsin prepared by autolysis of the mucous membranes of sheep stomach was effectively used for pilot scale enzymatic hydrolysis of fish solid waste with 50% specific yield.[29]

2.7.1.2 Functionality

Digestive enzymes and extracts exhibit uniform functionality and increased replicability because of their narrow cleavage-specificity. Salmon pectoral fin protein subjected to treatment with several enzymes (Alcalase, Flavourzyme, Neutrase, pepsin, Protamex, and trypsin), demonstrated peptic hydrolysate to possess the highest antioxidant properties.[79] A similar report using tilapia (*Oreochromis niloticus*) frame protein resulted in trypsin hydrolysate exhibiting the highest degree of hydrolysis and antioxidant activity.[84] Endogenous proteases from fish pyloric caeca has been used in the hydrolysis of skin gelatin with antioxidative properties of the resultant peptides on par with that produced with commercial enzymes such as Alcalase and Neutrase.[24] Mackerel intestinal crude enzyme was used by Je et al. (2005) to generate antioxidative hydrolysates from Alaska pollock (*Gadus chalcogrammus*) frame protein.[85] DH was demonstrated to exert a considerable influence on antibacterial activity of trout (*Oncorhynchus mykiss*) by-product hydrolysate prepared with trout pepsin.[86] In contrast, peptic hydrolysates of half-fin anchovy (*Setipinna taty*) demonstrated to have moderate or negligible metal chelating activities despite a high reducing capacity.[87]

2.7.2. Broad specific enzymes

Commercial microbial proteolytic preparations offer a distinct advantage in that, microbes are easy to culture and harvest. Microbial proteolytic systems are also very broad and variant, with a plethora of proteases available for use (Alcalase (*Bacillus licheniformis* Subtilisin A), Esperase (*B. lentus*), Neutrase (*B. amyloliquefaciens*), Flavourzyme (*Aspergillus oryzae*), Everlase (secreted from *Bacillus sp.*), Protamex (*Bacillus sp.*) and Savinase (*B. lentus*)). DH plays a major role in both protein recovery and functionality of protein hydrolysate. DH is influenced by a number of factors including preparatory conditions (pH, temperature, pretreatments), E/S ratio, enzyme type and protein substrate properties. However, contradictory results suggesting the relative insignificance of these factors are also in plenty. For example, properties of protein source although considered an important factor, was shown to have no influence (no statistically significant difference) over DH in a study that compares Whitemouth croaker (*Micropogonias furnieri*) muscle with other by-products, as source of proteins.[88]

2.7.2.1. Recovery during processing and efficiency

Linder et al. (1995) elucidated the effect of preparatory parameters such as initial protein content in the sample, temperature, pH and protease concentration during enzymatic hydrolysis (Neutrase) on the recovery of protein content from veal bone hydrolysates.[41] Effects of temperature (40-60 °C) and pH (5.5-7.5) was reported to be statistically insignificant, and duration of hydrolysis and enzymatic concentration played the most important roles in recovery.[41] Moreover, He et al. (2012) found E/S ratio, DH and processing time to influence protein recovery, whereas type of protease (Flavourzyme, Neutrase and Alcalase) used did not significantly influence protein yield from Atlantic salmon (*Salmo salar*).[89] Contradictory results

were also reported, where temperature of ~ 60 °C triggered the highest protein recovery with Alcalase proteolysis of Yellowfin Tuna (*Thunnus albacares*) viscera.[90]

Alcalase was found to be most efficient in protein recovery in general, for example, from Atlantic salmon (*Salmo salar*) by-products, among the hydrolysates generated by Alcalase, Promod and Protex, Alcalase proved be most effective in protein recovery.[91] Another report on Alcalase hydrolysis, achieved 92% protein yield was observed in the grass carp (*Ctenopharyngodon idella*) skin hydrolysate at a DH of 15%.[92] In contrast, a study found serine Atlantic salmon pyloric caeca enzyme extracts to be comparable to commercial alkaline proteases (Alcalase, Flavourzyme, Corolase) in terms of DH.[47] DH has been reported to remain constant and saturate after 120 mins for protein hydrolysates prepared with Flavourzyme.[88] Contrastingly, DH of 60% was observed in the case of protein hydrolysates prepared from round scad (*Decapterus maruadsi*) with Flavourzyme.[93] Protamex treatment resulted in the liberation of 48% of total protein in Atlantic salmon (*Salmo salar*) into the aqueous fraction (crude protein hydrolysate).[94]

From the bench scale to commercial production, protein recovery is expected to vary significantly. In fact, bacterial proteases when used to hydrolyze whole fish (*Mugil cephalus*) was only able to recover protein fractions between 13-15 % of input, in pilot scale, of which 83-86% was proteinaceous.[95] But, it was observed that an increase in the concentration of protease added to the processing resulted in a quadratic increase in soluble nitrogen.[95] As mentioned in an earlier section (2.3), the cost of enzymatic preparations have limited the development of enzymatically bioprocessed fish by-product protein hydrolysate.

2.7.2.2. Functionality

Šližytė et al. (2009) evaluated the influence of storage (of raw materials) and preparation on the yield, efficiency and functionality of cod backbone hydrolysates.[96] Fresh raw materials resulted in higher yields and better emulsification properties of the hydrolysate.[96] A high protein recovery (high DH) has been associated with bitter taste and reduced surface properties in salmon (*Salmo salar*) protein hydrolysates.[91] Additionally, longer processing also leads to higher DH and in turn, to better yields.[96] However, as with other processing approaches, increase in DH decreased water holding capacity of hydrolysates and did not influence their 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability or lipid peroxide inhibition in a liposome model.[96] Better interfacial properties of hydrolysates have been associated with increased chain length i.e., when DH is low.[7]

Skipjack Tuna (*Katsuwonus pelamis*) dark muscle protein hydrolysates prepared with Alcalase and Neutrase demonstrated higher antioxidant capacity as compared to hydrolysates prepared with pepsin, trypsin and papain.[27] In contrast, Patin (*Pangasius sutchi*) myofibrillar protein hydrolysates generated with papain had higher antioxidant properties (and higher DH) as compared to Alcalase hydrolysate.[97] Pollock skin protein hydrolysate prepared with Alcalase, applied as a glaze on salmon fillets have been shown to be effective in improving storage quality and limited lipid peroxidation.[98] Results from these studies verified that smaller molecular size, presence of hydrophobic and aromatic amino acid residues, and amino acid sequences are imperative in antioxidant activity. In addition to peptides, Belhaj et al. (2013) demonstrated that phospholipopeptidic complex obtained *via* enzymatic hydrolysis (Alcalase, Neutrase, and Flavourzyme) of salmon (*Salmo salar*) heads are able to exert anxiolytic-like effects on mice in a time and dose-dependent manner.[99]

2.7.3. Immobilized enzymes

Use of immobilized enzymes in an industrial setting offers several advantages over conventional enzymatic hydrolysis. Immobilized enzymes allow a more controlled and milder form of processing. Enzymes are expensive and it is economically sustainable to recycle enzymes. Immobilized trypsin, chymotrypsin, and carboxypeptidases on glioxyl-agarose supports was used to prepare *Brassica carinata* protein hydrolysates with a 36% DH.[100] Similarly, a column reactor filled with immobilized chymotrypsin–trypsin (I-CT) was used to prepare antioxidant peptides from hairtail surimi wash water.[101] Marine processing industries would benefit from the utilization of processing wastes such as shrimp head waste, consisting of extracted chitin and chitosan materials, which were employed to immobilize semi-purified acidic proteases from Monterey, sardine stomachs, another form of processing waste.[12]

2.7.4. Pre-treatment: Heat, ultrasound and microwave-assisted extraction

Physical pretreatment of protein concentrate or tissue homogenate has been utilized in several studies.[102–104] Ultrasound treatment incorporated processing of tilapia (*Oreochromis niloticus*) muscle protein resulted in lowered DH, whereas, reducing power and radical scavenging activity improved.[104] In contrast, microwave treatment of trout frame protein improved degree of hydrolysis, protein solubility and recovery, along with improved antioxidant activity.[102,105] Pre-treatments with physical methods listed above can result in the functional modifications of proteins/peptides, influencing their secondary, tertiary and quaternary structure.[103,106] In addition, pretreatments have been demonstrated to result in the formation of chemical conjugates such as the Maillard reaction products resulting from the glycosylation of proteins and peptides.[107] These treatments can be modulated and applied in addition to enzymatic

protein hydrolysis, either as a pretreatment or during processing for enhancing functionality and extractability of proteins and peptides.

2.8. Concluding remarks

A large section of the literature focuses on the solvent based approaches for the recovery of proteins or concentrate preparation from processing by-products. And among the different techniques used in the production of protein hydrolysates, it is clear that the use of proteolytic enzymes facilitates hydrolysate preparation with predetermined properties in a controlled manner. However, cost of enzymes has limited the application in the food processing industry. Microbial fermentation is also a potential approach that can be tailored for functional protein hydrolysates. In general, DH increases the yield by increasing the solvent accessibility of peptides. However, proteinaceous content recovered has been shown to vary all across the spectrum depending on the type of tissue and the processing method. Development of effective techniques for concentrate preparation, large-scale hydrolysis and recovery with good functional properties can lead to the sustainable and economical production of protein hydrolysates.

2.9. Objectives

Although several proteolytic approaches have been utilized in the production of fish-based hydrolysates from a wide range of substrates, along with characterization of functional properties. Reports focusing on marine by-products as sources for extraction of bioactive peptides generally tend to have longer and complicated processing steps (separate protein isolation and processing). Additionally, there still exists a gap between research comparing functional fish by-product protein hydrolysate generated *via* enzymatic, chemical and microbiological processes, and their practical commercial application. An extensive evaluation of yield and functional properties of chemical, microbiological and enzymatic proteolysis of

salmon (*Salmo salar*) by-products in an integrated processing approach, from the perspective of commercial production can thus, open further possibilities in utilizing these waste streams for functional food/feed development. This study primarily aims to simplify and integrate the steps involved in by-product protein hydrolysate preparation to ensure applicability during scale-up, while also focusing on developing physiologically relevant models and elucidating the mechanism of antioxidant activity of peptide preparations.

Therefore, the major objectives of this research work are,

- To evaluate the impact of bioprocessing on components of Atlantic salmon (*Salmo salar*) by-product fraction;
- To assess the effects of processing on the recovery of protein, oil, and residual fractions of the by-products;
- To characterize physicochemical properties of recovered by-product protein hydrolysate;
- To evaluate antioxidant properties of salmon by-product protein hydrolysate in physiologically relevant models.

CHAPTER 3

Yield, physico-chemical and antioxidant properties of salmon visceral hydrolysate: Comparison of lactic acid bacterial fermentation with Flavourzyme proteolysis and formic acid treatment

Abstract

This study compares a cost-effective microbial method, involving lactic acid (LA) fermentation, with the conventional formic acid (FA) treatment and Flavourzyme (FL) enzymatic hydrolysis for valorizing salmon processing waste (Viscera). LA and FA processing approaches relied upon production (LA) or addition (FA) of organic acids to lower the pH and activate the inherent proteases in these tissues, whereas FL processing was carried out at the optimum conditions of Flavourzyme (37 °C, pH 7.0). Highest protein hydrolysate fraction recovery (~57%) was seen in LA fermentation, while FA processing resulted in the highest protein levels (~87%) in the recovered hydrolysate fraction and lowest residual fraction (~4%). In general, FL demonstrated higher Fe(II) chelation (73%) and ferric reducing capacity (27 mM glutathione equivalent) whereas LA-hydrolysates showed enhanced sacrificial antioxidant properties. Findings from this study would have implications on the application of the processing approaches towards incorporation of salmon-based hydrolysates in food formulations, as well as help in understanding the role of various parameters in determining physiochemical and antioxidant properties of the hydrolysates.

3.1. Introduction

The fish processing industry is a large source of food waste in the form of by-products (heads, viscera, frame and skin), which are currently, largely underutilized, despite their high content of food-grade proteins. Commercial-enzymatic approaches, although expensive, are gaining ground for the preparation of fish by-product hydrolysates.[6,7] Autolytic methods, on the other hand, are cheaper and more sustainable than commercial-enzymatic preparations, but are generally considered to be ineffective for the production of hydrolysates with specific functionalities.[8] The array of endogenous enzymes in the fish tissues is also known to change with season, age, gender of the fish and other environmental factors, and would also vary within and between species.[8]

Owens and Mendoza have extensively reviewed the preparation of a number of fish-based products that utilize enzymatic hydrolysis, autolysis and bacterial fermentation.[5] Fish sauce is one of the major products worldwide that utilizes fermentation, or slow hydrolysis in a high salt concentrated slurry of minced fish. According to Kristinsson and Rasco, fish sauce production is generally done at neutral pH and acid-dependent proteases play a relatively minor role in the process.[8] Slow extensive breakdown of the fish muscles takes place with the help of endogenous serine proteases. Contrastingly, fish silage production, although similar in certain characteristics with fish sauce preparation, is more rapid, occurs at an acidic pH and is targeted towards use in animal feed and not human consumption.[8] There is limited research on the use of an autolytic approach for the production of protein hydrolysates intended for human consumption. Commercial proteases, as mentioned earlier, are predominantly used in hydrolysate preparation. Enzymatic hydrolysis of fish by-products, utilizing proteases of plant, animal or microbial origins, has facilitated the tailoring of proteolysis based on the desired

product functionality.

Although autolysis has been utilized in the production of fish-based hydrolysates, functional properties have not been extensively evaluated or compared to commercial enzymatic hydrolysate preparations. There still exists a gap between research on functional protein hydrolysates and commercial application. The objective of this work was to characterize the physicochemical changes during different bioprocesses and also determine their effect on antioxidant activity of the protein hydrolysate from salmon viscera, a reservoir of endogenous enzymes. The study aims to simplify and integrate the protein hydrolysate preparation, extraction and processing steps to ensure applicability during scale-up.

3.2. Materials and Methods

3.2.1. Preparation of Slurry

The by-products were obtained from market size Atlantic salmon (*Salmo salar*). The offal was procured from a local gutting plant (harvested fish processed to HOG (Head on Gutted)) where it was immediately frozen after processing. The offal was thawed (in room temperature), mixed and blended (Ninja® Professional Blender BL660C, SharkNinja Operating LLC, QC, Canada) to form a uniform semi-solid mix. Blended mix was then stored at -20 °C till further processing. For the different treatments, 750 g of the slurry was withdrawn and added to a sealed batch fermentor (New Brunswick™ Bioflow®/CelliGen® 115, GMI Inc., MN, USA).

3.2.2. Processing Approaches

All three processing approaches used in this study involve “solid state production”, with no additional/free water added to the ground viscera i.e. the moisture in the ground viscera originated from the visceral tissues itself.

3.2.2.1. Formic Acid (FA) Treatment

Formic acid (88 %, Thermo Fisher Scientific, ON, Canada) aqueous solution was added (2% w/w) to the homogenous visceral slurry (modified from Vieira et al., 2015).[108] The temperature in the fermentor was maintained at 37 °C and agitated continuously at 150 rpm. Samples were withdrawn at 0, 0.25, 0.5, 1, 2, 4, 6 and 8 days and stored at -20 °C for further analysis.

3.2.2.2. Lactic Acid (LA) Fermentation

Deproteinized whey 5% (w/w), (80-90% lactose, obtained from Saputo Inc., Montreal, Canada) and 1% 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)) (LALFEED® LACTO, Lallemand Inc., Aurillac, France) were mixed thoroughly with the ground viscera. Temperature and stirring conditions were maintained under the same condition as FA treatment in the fermentor. Samples were withdrawn at the above-mentioned time points and stored at -20 °C until further analysis.

3.2.2.3. Flavourzyme Proteolysis (FL)

The protease obtained from *Aspergillus oryzae*, Flavourzyme (Sigma-Aldrich, ON, Canada, EC. 232-752-2) was mixed with the slurry in the enzyme-to-substrate ratio of 1:100 (w/w). pH was monitored and maintained automatically at 7.0 with 0.1 M NaOH, and hydrolysis was carried out under the same temperature and stirring conditions as the FA and LA processing (under the optimal temperature condition (37 °C) for FL). FL proteolysis was carried out for a relatively short period of 12 hours since increasing the hydrolysis time beyond 12 hours led to fouling of the visceral mix. Samples were withdrawn at 0, 3, 6 and 12 hours (0.0, 0.125, 0.25 and 0.5 days, respectively) and stored at -20 °C.

3.2.3. Characterization of the Bioprocessing Approaches

3.2.3.1. Fractionation and Yield

Samples (8 g) collected at different time intervals (mentioned above) were transferred to weighed tubes and centrifuged (3,500×g) for 15 min. The different fractions were then collected into weighed tubes and lyophilized. Both wet and dry weights were measured and used to calculate the yield.

3.2.3.2. Lactose/Lactic Acid Determination

Samples collected at different time intervals from LA fermentation were diluted 100 times in ultrapure water and centrifuged at 15,000×g for 10 min and clear supernatant was collected. Lactose/lactic acid content was determined using a Flexar high performance liquid chromatography (HPLC) system equipped with a refractive index detection (PerkinElmer, Shelton, CT, USA). The chromatographic separations were performed on an Aminex HPX-87H (300 mm × 7.8 mm, 9 µm) ion exclusion column (Bio-Rad Laboraties, Hercules, CA, USA) at 35°C. Injected samples (20 µL) were eluted in an isocratic mode with 0.008N sulfuric acid solution using a flow rate of 0.5 mL/min for 30 min. The quantification of lactose and lactic acid was achieved using a standard curve after integration of peak areas.

3.2.3.3. Colony Count to Estimate LAB Growth

Serially diluted (1×10^{-7}) samples collected at 0, 0.25, 0.5, 1, 2, 4, 6 and 8 days' interval was spread (100 µL) on DeMan-Rogosa-Sharpe (MRS) agar (Sigma-Aldrich, MO, USA) plates (Incubated at 37 °C for 2 days) to estimate the changes in growth of LAB over the course of the LA fermentation. Results were expressed as colony forming units per mL (cfu/mL).

3.2.3.4. Protease Activity over Time

Protease activity within the visceral slurries was performed with hemoglobin as a

substrate (Modified from Ichishima and Yoshida (1963)).[109] Samples withdrawn at different time points were diluted 6 times in d.H_2O (0.5 g (sample) + 2.5 g (d.H_2O)) and centrifuged (3,500×g) for 15 min. The soluble fraction was carefully withdrawn (50 µL) and added to 250 µL of 2.5 % (w/v) hemoglobin (Sigma-Aldrich, ON, Canada) solution in d.H_2O (pH 4.5). To subtract matrix effects (peptides/amino acids from sample and hemoglobin), a blank (without the sample) and background (without hemoglobin) were also prepared. Following incubation at 37 °C for an hour, 500 µL of 100 mM trichloacetic acid (TCA, Sigma-Aldrich, ON, Canada) was added and the mixture was maintained at room temperature (RT) for 30 min. The mixture was centrifuged (15,000×g) for 15 min (25 °C) and 50 µL of supernatant was transferred to 96-well plates. Sodium carbonate (0.5 M, 125 µL) and 25 µL of Folin-Ciocalteu's phenol reagent were also added to the wells and incubated for 30 min (RT). The absorbance was read at 660 nm using a microplate reader (Tecan M1000, Männedorf, Switzerland) and the values were plotted in terms of µmoles of tyrosine equivalent liberated using a tyrosine standard curve.

The units of protease activity were calculated using the equation:

$$\text{Units / mL} = \frac{(\mu\text{mole of Tyr} \times \text{Reaction volume (0.8 mL)} \times \text{dilution factor (6)})}{(\text{sample volume (0.05 mL)} \times \text{reaction time (60 min)} \times \text{volume assayed (0.05 mL)})}$$

The actual protease activity in the sample at different time points (U) was calculated from this after subtracting the effects of the matrix (hemoglobin and peptides present in the sample), i.e.

$U = (A - (H+S))$

Where, U is the actual protease activity in units/mL; A, protease activity of the sample with the matrix effects; H was calculated from the blank (hemoglobin alone), whereas S is the background (sample without the addition of hemoglobin).

3.2.4. Characterization of the Hydrolysates

Salmon visceral protein hydrolysates were prepared by centrifuging (3,500×g) the slurry (collected at different time intervals mentioned above) for 15 min. The liquid hydrolysate fraction was collected separately and freeze dried. The protein hydrolysate was then resuspended in d.H_2O at desired concentration for different analysis.

3.2.4.1. Degree of Hydrolysis

Degree of hydrolysis was calculated based on the method reported by Nielsen et al., 2001.[39,110] Sample (1 mg/mL, 33 µL) was added to 250 µL of O-phthalaldehyde reagent and the absorbance was measured at 340 nm.

3.2.4.2. Lowry Protein Estimation

Pierce™ Modified Lowry Protein Assay Kit (ThermoScientific™, ON, Canada) was used according to the manufacturer's instructions to determine the protein content of samples at 1 mg/mL concentration. Absorbance measured at 750 nm was plotted on a bovine serum albumin standard curve to determine the protein content of the samples obtained at different time points.

3.2.4.3. Determination of Surface Hydrophobicity (S_o)

8-Anilino-1-naphthalenesulphonic acid (ANS, Sigma-Aldrich, ON, Canada), a hydrophobic probe, was used in the determination of S_o.[111] Sample (0.0300% - 0.0009%, 100 µL) was mixed with 100 µL of ANS (0.04 mM) in a 96-well black plate. Fluorometric determination was done at excitation and emission wavelengths of 390 nm and 470 nm, respectively. The slope of the fluorescence intensity vs. concentration plot was calculated to be the S_o value.

3.2.4.4. Free Thiol Determination

Sulfhydryl (SH) groups were determined using DTNB [5,5′-dithiobis-(2-nitrobenzoic acid), Sigma-Aldrich, WI, U.S.A.].[112] Sample (150 µL, 4 mg/mL) was added to 50 µL of DTNB (3 mM, sodium phosphate buffer, pH 8.0) and incubated for 5 min at RT, and absorbance of the mixture was then measured at 412 nm. Glutathione (GSH) was used to obtain a standard curve and the SH content of the hydrolysates was expressed as µM GSH equivalent.

3.2.4.5. Fluorometric Determination of Intermediate Maillard Reaction Products (iMRPs)

The presence of iMRPs was detected fluorometrically (at final concentration of 2 mg/mL) with a microplate reader (Tecan M1000, Gain set at 194) at excitation and emission wavelengths of 347 nm and 420 nm,[39] respectively.

3.2.4.6. Fluorometric Detection of Lipid Peroxides

Diphenyl-1-pyrenylphosphine (DPPP, Molecular Probes™, OR, U.S.A.) was used as a fluorescent probe to detect levels of lipid peroxides in the hydrolysates prepared from the slurry at different time intervals. DPPP reacts with lipid peroxides to give DPPP oxide which was detected fluorometrically at excitation and emission wavelengths of 361 nm and 380 nm respectively.[113] DPPP (50 µg/mL, 50 µL, dissolved in dimethyl sulfoxide (DMSO) and diluted using d.H_2O) was added to 150 µL of sample (4 mg/mL) and incubated for 5 min (RT), and fluorescence intensity of the assay mixture was then measured.

3.2.5. Antioxidant Assays

3.2.5.1. Metal Chelation

Metal chelation assay was performed based on a method by Saidi et al. with modifications.[114] Sample (4 mg/mL, 500 µL) was equilibrated with 25 µL of $FeCl_2$ (2 mM, Sigma-Aldrich, ON, Canada) for 10 min (RT). Following equilibration, 50 µL of FerroZine™ (5

mM, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*′-disulfonic acid monosodium salt hydrate, Sigma-Aldrich, ON, Canada) was added and incubated for another 10 min (RT). The mixture (200 µL) was then transferred to a 96-well plate and absorbance was measured at 562 nm.

3.2.5.2. Ferric Reducing Antioxidant Potential

The ferric reducing antioxidant potential (FRAP) of the hydrolysates was carried out based on a method by Mohan et al.[39] Equal volumes (100 µL) of sample (4 mg/mL) and potassium ferricyanide (1% w/v, Sigma-Aldrich, ON, Canada) were incubated at 50 ºC for 20 min. 100 µL of TCA (10% w/v) was added and vortexed. The mixture was centrifuged at 8,000×g and 100 µL of the supernatant was transferred to a 96-well plate containing 80 µL of d.H_2O. Thereafter, 20 µL of $FeCl_2$ (0.1% w/v) was added and the mixture was incubated for 10 min (RT) and absorbance was measured at 700 nm. The ferric reducing antioxidant potential was expressed as mM GSH equivalent based on a GSH standard curve.

3.2.5.3. Glutathione (GSH) Protection

The sacrificial antioxidant capacity of the hydrolysates was assessed using a GSH protection assay modified from Udenigwe et al.[115] GSH (80 µg/mL, 300 µL, Sigma-Aldrich, ON, Canada) was equilibrated with 125 µL of sample hydrolysates (4 mg/mL) for 15 min at RT followed by the addition of 50 µL of $FeCl_2$ (1 mM) and 25 µL of H_2O_2 (20 µM). The mixture was vortexed and incubated at 37 °C for 90 min. Following incubation, protected GSH was estimated using DTNB following the same protocol as thiol determination.

3.2.6. Statistical Analysis

All the analyses were conducted in triplicates and the results were expressed as mean ± std. deviation. Two-way analysis of variance (ANOVA) was performed followed by Tukey's HSD test for the separation of means using SAS® university edition (SAS Institute Inc., Cary,

NC, USA) along with the macro, pdmix800.[116] Spearman's rank order coefficient (ρ) was calculated to determine correlation between different variables using Sigmaplot 12.1 (Systat Software, San Jose, CA, USA).

3.3. Results and Discussion

The ground salmon viscera used in this study was susceptible to liquefaction due to large amounts of inherent proteases, and liquefaction occurred spontaneously on sample thawing. This is possibly a result of increased activity of collagenolytic enzymes, which have been shown to increase with temperature.[60] There are several advantages to using a lactic acid bacterial fermentation system instead of formic or other organic/mineral acids for hydrolysis of fish by-products. LAB are cheap to culture and maintain, with lower operational costs compared to the acid approach which involves purchase and handing of acids.

3.3.1. Fractionation and Yield

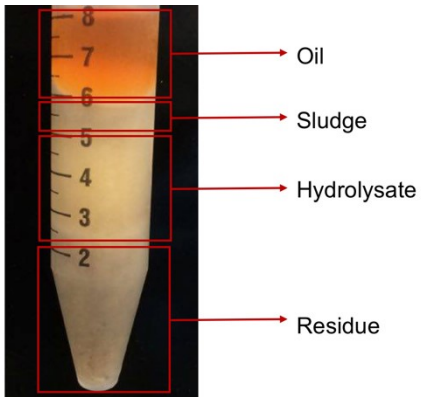

Figure 3. 1. Centrifugally fractionated slurry of the bioprocessed salmon viscera.

The slurry of hydrolyzed viscera, when centrifuged, separated into four distinct fractions (**Figure 3.1**) similar to a previous report on salmon by-product processing.[46] As hydrolysis proceeds, the levels of the residue (containing ash, insoluble proteins, bone remnants) was

expected to decrease as more of the structural proteins are broken down.[45] As predicted, DH consistently correlated negatively (not significant) with the yield of residual fraction in all three bioprocessing approaches (LA, $\rho = -0.929$, $p = 2\times10^{-7}$, $n = 8$; FA, $\rho = -0.595$, $p = 0.102$, $n = 8$; and FL, $\rho = -0.400$, $p = 0.750$, $n = 4$). Lower values of the residual fractions (~4%, **Figure 3.2. A**) were observed in FL and FA processing at time points with relatively higher DH.

As mentioned earlier, intact structural proteins that hold together the salmon tissues would be broken down to release peptides along with moisture as protein hydrolysis proceeds. As predicted, the wet yield of the soluble/hydrolysate fraction (**Figure 3.2. B**) increased significantly (LA, FA and FL) with time, saturated and then decreased (LA, FA; not statistically significant). In addition, an inverse relation is observed between the emulsion/sludge fraction (containing a complex of lipids and proteins) relative to the hydrolysate fraction (LA, $\rho = -0.548$, $p = 160$, $n = 8$; FA, $\rho = -0.214$, $p = 0.578$, $n = 8$; and FL, $\rho = -1.000$, $p = 0.083$, $n = 4$). This sludge fraction has been demonstrated to be composed of partially hydrolyzed proteins in Flavourzyme hydrolyzed cod (*Gadus morhua*) by-products.[117]

Increase in sludge fraction during LA fermentation over time can be attributed to emulsification of fats and complex of proteins, peptides and lipids formed/liberated.[46] Characterization of the phospholipopeptidic complex formed during enzymatic hydrolysis of Atlantic salmon heads found these to be composed of phospholipids, triacylglycerols, peptides and minerals.[99] Moreover, peptidic fraction of the phospholipopeptidic complex formed in the sludge fraction of enzymatic hydrolysis of Atlantic salmon heads was assessed using nano-LC LTQ Orbitrap XL.[99] It was found that peptides (4.2 kDa – 13.2 kDa) originating from collagen and myosin isoforms were predominantly present in these fractions.[99] Contrary to LA fermentate, both FA and FL hydrolysis resulted in a decrease in the sludge fraction over time, although, the

sludge content values (%) were significantly different between FA treatment and FL hydrolysis. Additionally, both FL and LA yielded far less oil content as compared to FA. It is predicted that the oil was complexed/emulsified forming the sludge layer in these approaches. However, the mechanism underlying FA and FL processing and reduction (FL) or increase (LA) in the sludge fraction over time is still not clear. The oil fraction obtained at different time points from FA treatment increases with time and then decreases. The highest oil yields (21%) from salmon were on par with previous studies of fish oil derived from autolysis approaches.[118]

It is noteworthy to mention that the yield of the fractions was significantly different even at the earliest time points. This could be because of the structural biomolecules in these tissues behaving differently at different pH (LA - 6.09, FA - 3.35, FL - 7.00).

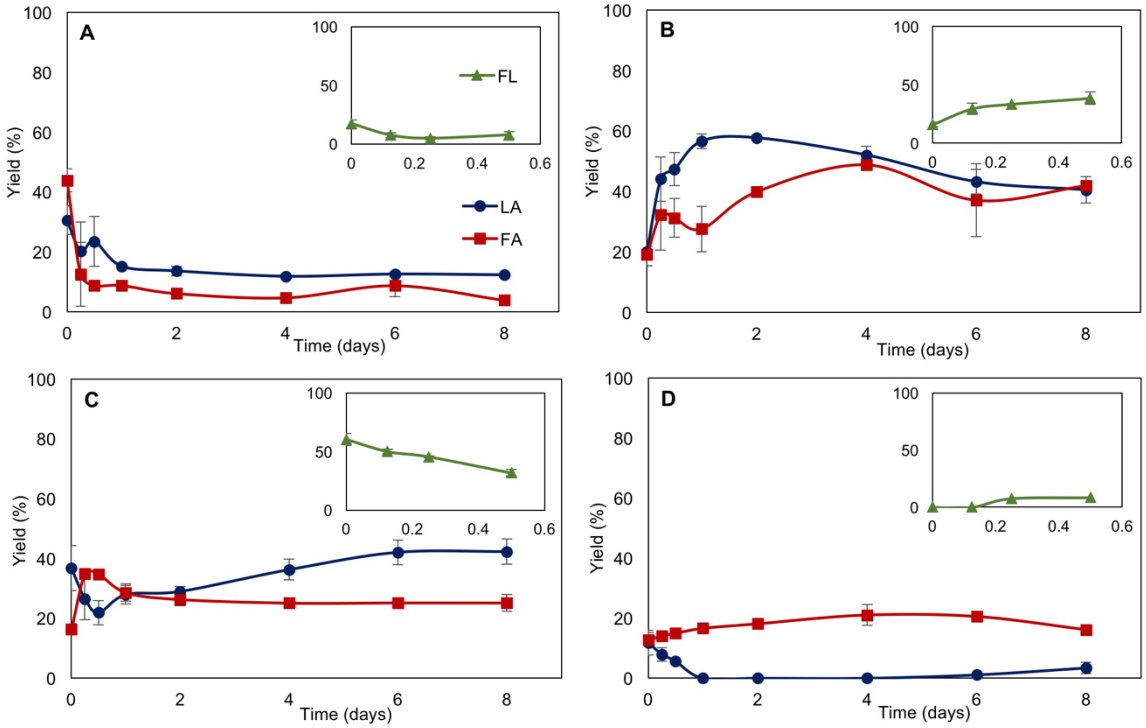

Figure 3. 2. Change in yield (wet weight basis) of the (**A**) Residue, (**B**) Hydrolysate, (**C**) Sludge, and (**D**) Oil over time for LA, FA and FL.

3.3.2. Characterization of bioprocessing

FL was chosen from among the several commercial enzymatic preparations in this study, as the optimum temperature for FL activity is the same as FA and LA treatment. Moreover, FL has been demonstrated to release peptides with strong antioxidant potential.[7] Therefore, all the treatments were carried out at the same temperature and stirring conditions.

The consumption of lactose by the LAB was at a slower rate (F**igure 3.3. A**) compared to the growth and decline of LAB population (**Figure 3.3. C**) The rapid decline seen in LAB populations can thus be attributed to the unfavourable pH range of the LAB visceral fermentate. Although further microbial studies would be needed to establish the status of any harmful-microbial development, previous studies with LA-fermentate stabilized at pH less than 4.2 have demonstrated substantially reduced populations of *Staphylococcus*, *Clostridium* and coliform bacteria as compared to raw fish by-products obtained after processing.[65,66]

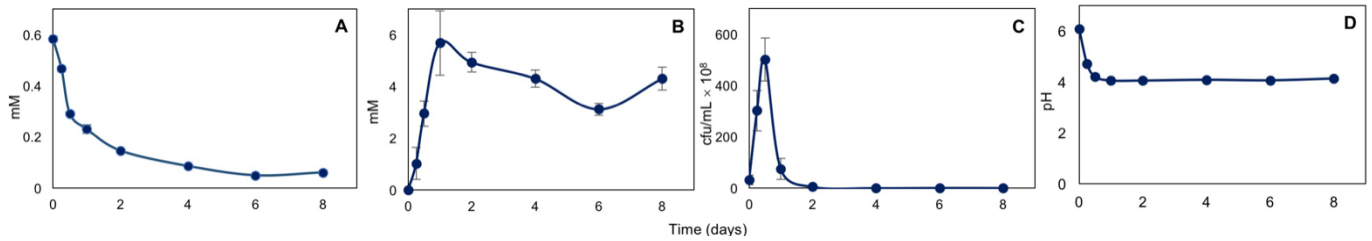

Figure 3. 3. Characterization of the lactic acid (LA) fermentation process: (**A**) Change in lactose concentration over time as quantified with HPLC. (**B**) Lactic acid concentration during fermentation. (**C**) Lactic acid bacteria (LAB) populations at different time points of fermentation as estimated through colony counts. (**D**) Change in pH during LA fermentation.

The pH steadily declined along with rapid accumulation of lactic acid with time and then stabilized at around 24 hours (**Figure 3.3. B and D**). Similarly, change in pH has been observed, albeit at a lower magnitude, for the first 5 days of fermentation of squid by-products with *Aspergillus oryzae*.[44] The metabolic and growth parameters of LAB in fish by-product based

media has been previously assessed by Vázquez et al. (2008).[119] It is possible that the decline seen in lactic acid concentration (**Figure 3.3. B**) after attaining a peak is as a result of utilization of lactic acid by certain bacteria to produce butyric acid or acetic acid.[120] In fact, *Lactobacillus plantarum* and *Lactobacillus buchneri* have been characterized to generate a significant dip in the levels of lactic acid, after reaching a maximum with an increase in acetic acid concentration in a media supplemented with fish visceral hydrolysate.[119] The pH change during the FA treatment on the other hand, remained between 3.35 and 3.50 without significant difference.

Salmon tissues, like most other living tissues is mainly composed of water which is entrapped by the structural components of the tissue. Addition of water and its removal are barriers for the final protein hydrolysate product not reaching the marketable quality.[8] All three approaches utilized here do not require addition of water, and utilized the water content of the salmon tissues only. The approaches here, bearing similarities to solid state fermentation (SSF) ensures that no additional free water is added to the processing batch. Moisture/water, along with the peptides was released as proteins (structural units in tissues and organs) were degraded by proteases (endogenous/exogenous). Moisture content in the hydrolysate fraction (**Figure 3.4.**) was reflective of the protease activity (**Figure 3.5.**) observed, especially in the initial time points of the different bioprocessing approaches. A similar but elaborate and expensive approach has been utilized for the SSF of *Acanthogobius hasta* with *Aspergillus oryzae*.[69] However, most of the previous studies extracted fish by-product protein hydrolysate following the addition of water to the slurry/fermentate.[46,69]

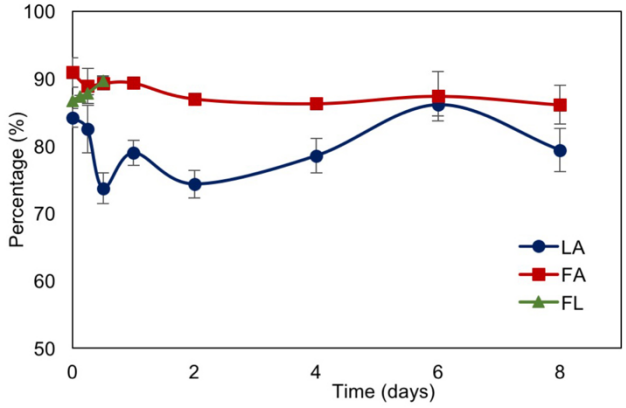

Figure 3. 4. Change in moisture content of the hydrolysate fraction over time for the different processing approaches.

3.3.3. Protease Activity during processing

FA treatment and LA fermentation are reported as autolytic approaches in the literature,[8] i.e., low pH induced by the addition of formic acid and production of lactic acid, respectively, is expected to increase endogenous proteolytic activity. However, formic acid (2% v/v) has been reported to induce chemical cleavage of proteins at the aspartyl residues,[59] and this property is currently being utilized in protein identification.[59] Additionally, proteolytic activity of LAB strains have been characterized,[61,121] and LAB are reported to weakly exhibit proteinase, aminopeptidase and peptidase activity.[61] However, the protease activity in LA fermentation does not correspond with the growth and decline of LAB populations (**Figure 3.5.)**. Therefore, LAB secretory enzymes were inferred not to play any significant role in proteolysis.

As expected, it was observed here that the enzyme activity significantly increased with decrease in pH in the case of LA fermentation (**Figure 3.5.)**. In contrast, previous studies have shown that collagenolytic activity of salmon increases significantly with pH (enzyme activity at pH - 6.00, 8.00 > pH - 4.00).[60,122] In the present study, it was predicted that the endogenous

enzymes (pepsin and catheptic proteases) are activated or liberated as a result of lowered pH which drives forward hydrolysis in both FA treatment and LA fermentation.

In the case of FL hydrolysis and FA treatment, there is a significant drop in enzyme activity over time (**Figure 3.5.**), which can be attributed to the enzyme degradation over time (autodigestion),[95] modification of enzymes and peptides affecting enzyme performance.[123] Conversely, for LA fermentation, the enzyme activity increases with decrease in pH until the 6th day, following which the activity decreases for the same reasons mentioned above.

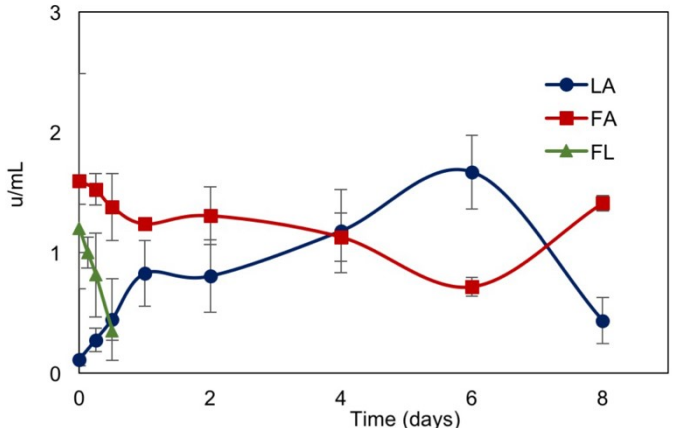

Figure 3. 5. Change in protease activity (U/mL) at different time points for the three different bioprocessing approaches; lactic acid (LA) fermentation; formic acid (FA) treatment; Flavourzyme (FL) hydrolysis.

3.3.4. Characterization of hydrolysates

3.3.4.1. Degree of hydrolysis (DH)

The change in DH was not statistically significant for FA-induced autolysis after time point zero, indicating rapid hydrolysis immediately following the addition of FA to the fish by-product slurry (**Figure 3.6. A**). In contrast, proteolysis during LA fermentation proceeded slowly with DH increasing significantly until the 4th day of fermentation.

In FL proteolysis, following the initial increase in DH at 3 hours, no statistically significant differences were observed for FL-hydrolysates from 6 hours and 12 hours (**Figure 3.6. A**). As a matter of fact, DH has been demonstrated previously to stabilize after 2 hours for Flavourzyme proteolysis.[88] The DH values were expected to correspond with the enzyme activity data; however, it is seen that FL hydrolysis, which displayed the lowest enzyme activity had the highest DH (**Figure 3.6. A**). The discrepancy observed here can be as a result of a change in pH from 7.0 (the optimum of Flavourzyme activity) to 4.5 (pH of hemoglobin), at which the enzyme activity assay was carried out.

3.3.4.2. Protein Content in the Hydrolysate Fraction

Around 80% of the proteins in the fish tissue are reported to be solubilized in a week during silage production.[9] Similarly, high protein contents (87.78% of the dry weight) was achieved in FA treatment at 4 days (**Figure 3.6. B**). FA treatment was observed to have the highest levels of protein in the lyophilized crude hydrolysate fractions followed by FL-hydrolysate, and LA-hydrolysates had significantly less protein content as determined by modified Lowry assay. Dilute formic acid is an established solvent for protein solvation and extraction (especially for mass spectrometric analysis).[52,124] Proteins subjected to formic acid exposure (at room temperature) are susceptible to chemical modification (formylation).[52] The impact of formylation on meal, feed or food quality is yet to be assessed.

The observed values for protein content did not correspond to the magnitude of their respective DH values (**Figure 3.6. A**), although positive ρ values (not statistically significant) were observed. Even though hydrolysis resulted in slight increase in the recovered protein levels, merely breaking the protein complexes and increasing the solvent accessibility alone did not enhance protein hydrolysate recovery from salmon viscera.

3.3.4.3. Surface Hydrophobicity (S_o)

Kristinsson and Rasco described that hydrolysis of peptide bonds can result in a concomitant increase in S_o as a result of exposure of hydrophobic cores.[8] In LA and FA processing, which have slower proteolysis rates, an initial increase in S_o was observed, followed by a dip and saturation (**Figure 3.6. C**). Contrastingly, with exogenous FL hydrolysis, a negative relationship was observed between DH and S_o.[115] For FL, S_o steadily declined with an increase in DH (ρ = -1.000, p = 0.0833, n = 4). Based on the results presented here and in previous studies,[10,39] it is evident that change in S_o during hydrolysis depends on the nature of the protein substrate, chain length of peptides generated, intermolecular peptide interactions, and cleavage properties of the protease. However, the chemistry underlying the higher S_o values seen in FA-hydrolysates as compared to LA-hydrolysate remains unclear.

3.3.4.4. Thiol Content

The redox active SH of the hydrolysate has been implicated in ferric reducing capacity of protein hydrolysates. SH content was observed to decrease initially and then increase to the same level in all three bioprocessing approaches (**Figure 3.6. D**). Protein hydrolysis has been associated with increased SH contents[125] but the opposite has also been reported.[39] Contrary to the results from our previous report,[39] iMRP formation during the bioprocessing appears to not interfere with the increase in SH content of the hydrolysates. Additionally, the rate of increase in SH has been associated with higher levels of DH[39] and results presented here (**Figure 3.6. D**) follow a similar trend.[126] It is also noteworthy to mention that change in SH content did not correlate well with other measurements of antioxidant properties of the hydrolysates assayed here.

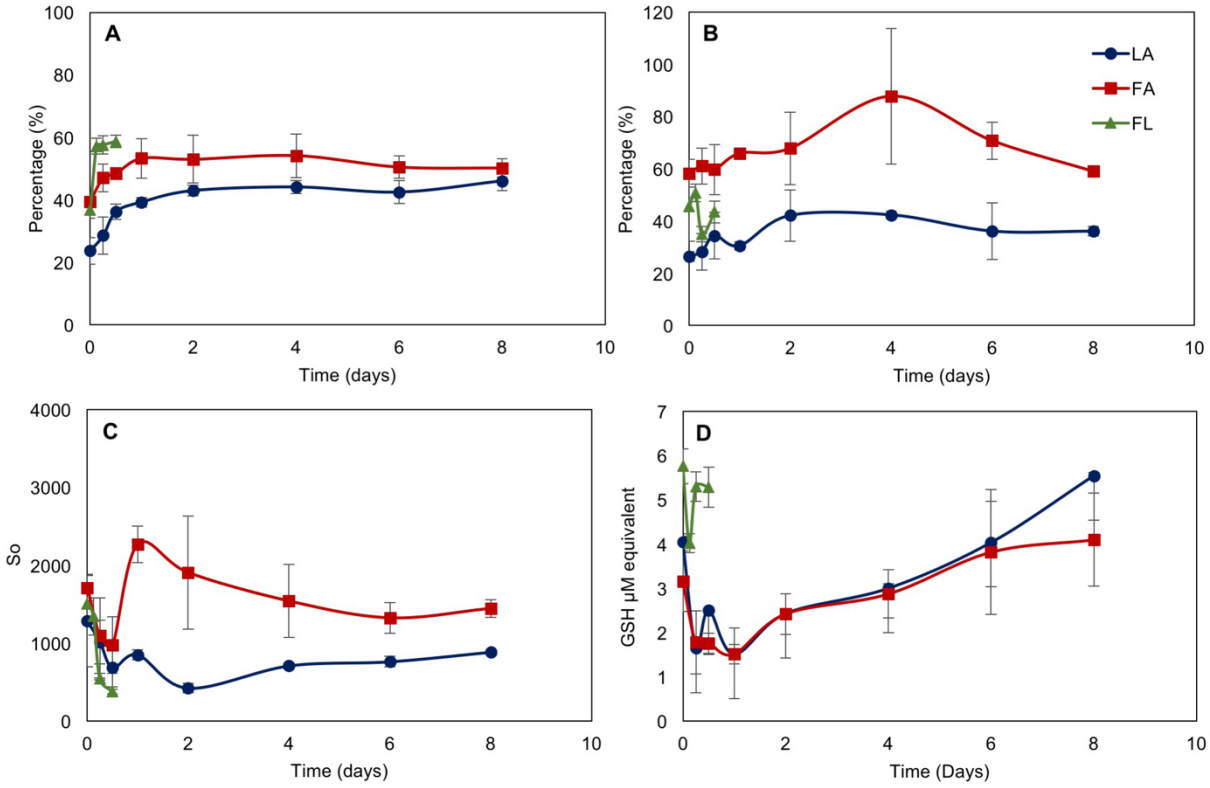

Figure 3. 4. Physiochemical characterization of the hydrolysate fractions collected at different time points for FA, LA and FL: (**A**) Degree of hydrolysis (DH, %), (**B**) Protein content (%) estimated through modified Lowry assay, (**C**) Surface hydrophobicity (S_o), and (D) Sulfhydryl concentration expressed as µM glutathione (GSH) equivalent.

3.3.4.5. Generation of iMRPs

Maillard reaction occurs as a result of reducing sugar moieties reacting with the free amino groups of peptides, free amino acids or proteins (pH and temperature dependent) resulting in the formation of Amadori products, which degrade and begin a chain of reactions that form intermediate and advanced MRPs.[127] Fluorescent-active iMRPs in the salmon by-products were found to be higher at zero time point for FL compared to LA and FA. In contrast, low iMRPs levels were observed at initial time points of FA treatment (**Figure 3.7. B)**. The disparity observed between FL, LA and the FA treatments can be explained on the basis of difference in

pH, which was low (3.35) at time point zero in the case of FA treatment. It has been established that higher pH helps to drive the condensation reaction between the sugar moiety (which would exist in open chain conformation at higher pH (>7.0)) and amino group of the peptides thereby increasing the levels of MRPs.[127] In both LA and FA processing, a marked increase in iMRPs was observed over the long processing duration of 8 days under acidic pH. In contrast, FL hydrolysis was carried out for 12 hours under a favourable pH condition for Maillard reaction, during which the iMRPs generated initially would be degraded or have formed advanced products. It is also noteworthy to mention that the color of the hydrolysates can be considered to be an important aspect for acceptability in food applications. The hydrolysates produced in this study had slightly creamy yellowish color. In contrast, fermentation with *Bacillus subtilis* was reported to yield brownish hydrolysates,[43] which is a result of higher levels of MRPs generated as a result of autoclaving, and a higher processing pH of 8.0.

3.3.4.6. Lipid Peroxide Content

Lipid peroxides are of great concern in food products including food additives, as it is associated with reduced shelf life and consumption (nutritional and organoleptic) quality. Although not statistically significant, a negative correlation was observed between the yield of oil at the different time points and lipid peroxidation, indicated by ρ values (LA, $\rho = -0.634$, $p = 0.071$, $n = 8$; FA, $\rho = -0.619$, $p = 0.086$, $n = 8$; and FL, $\rho = -0.949$, $p = 0.083$, $n = 4$). It is also apparent from **Figure 3.2. D** and **Figure 3.7. A** that lipid peroxides were significantly higher at time points with nil oil yield (observed at certain time points of LA and FL) as opposed to higher oil yields (FA). These results assert the importance of removal of lipids in the downstream processing of protein hydrolysates. Variation in the composition of emulsifying peptides or peptide clusters within the complex hydrolysate fraction, may have influenced the composition

of oxidation-prone fatty acids, at each time point during bioprocessing of the salmon viscera. It can be assumed that the total lipid content would remain the same during the processing of the fish slurry.

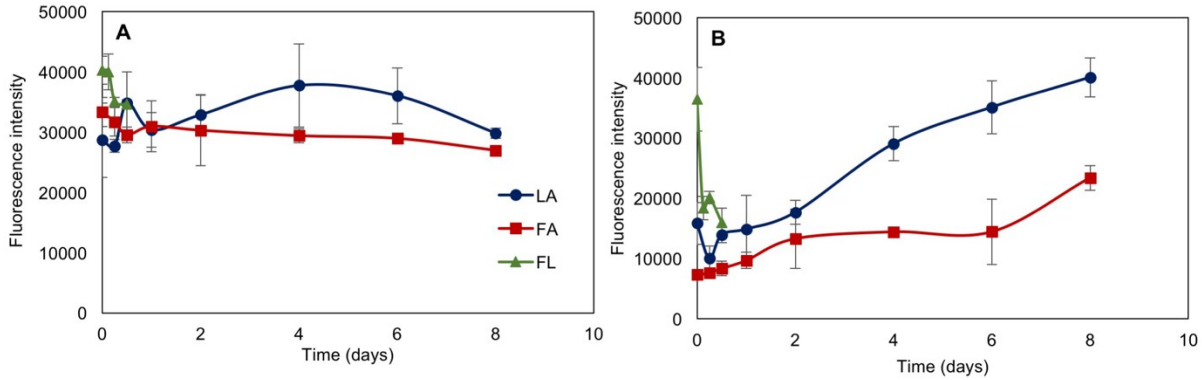

Figure 3. 5. (A) Quantification of lipid peroxides present in the salmon visceral hydrolysates withdrawn at different intervals measured as fluorescence intensity, (B) Generation of intermediate Maillard reaction products (iMRPs) during the bioprocessing measured as fluorescence intensity.

3.3.5. Antioxidant activity

3.3.5.1. Metal (Fe(II)) Chelation Activity

In terms of percentage of bound Fe(II) in this study, the highest Fe(II) chelating activity, observed in FL-hydrolysate withdrawn at 12 hours, can be expressed as 79.71±0.94% at a final hydrolysate concentration of 1.33 mg/mL, which is on par with previous reports.[7,128] Flavourzyme has been shown to generate hydrolysates with high metal chelation capacity.[7] Yellow stripe trevally (*Selaroides leptolepis*) meat, hydrolyzed with Flavourzyme had higher metal binding activity and reducing power than that hydrolyzed with Alcalase.[7] Contrastingly, flaxseed protein hydrolysate generated with Alcalase had higher Fe(II) chelation activity than the hydrolysate from Flavourzyme proteolysis.[128]

The results presented in this study (**Figure 3.8. A**) describe a decrease (following an initial increase in metal chelation activity (possibly as a result of optimal DH)) in both reducing capacity and metal chelation for the LA-hydrolysates with an increase in DH. The metal chelating capacity of FL-hydrolysates, on the other hand, remained unchanged even with the increase in DH (**Figure 3.8. A**). In contrast, Klompong et al. observed that increase in DH during enzymatic hydrolysis was accompanied by an increase in metal chelation whereas the reducing power of the hydrolysates remained unchanged.[7] Even though smaller peptides have tended to show higher Fe(II) chelation activity,[7] the contrary was observed in both LA- and FA-hydrolysates. Although peptic hydrolysates of half-fin anchovy (*Setipinna taty*) had moderate or negligible metal chelating activities.[87] As particular ligands are needed for the formation of strong metal coordinates, the difference in trend is attributable to the functionality and compositional heterogeneity, especially with regards to the cleavage pattern of peptides constituting the salmon viscera hydrolysates, which are expected to vary over the different bioprocessing approaches and time points.

3.3.5.2. Ferric Reducing Capacity

FL-hydrolysates demonstrated significantly higher Fe(III)-reducing capacity than the LA hydrolysate (**Figure 3.8. B**). The reducing capacity of the FL-hydrolysates are comparable to the values attained for commercial-enzymatic hydrolysates prepared from other protein isolates,[39,129] even though this work utilizes a cheaper alternative of minced fish slurry as the protein source. Similar results have also been demonstrated with fish meat hydrolysate prepared by fermentation with *Bacillus subtilis*.[43]

It is noteworthy to mention that the reducing capacity of LA-hydrolysates steadily decreased with the concentration of LA in the hydrolysate. Additionally, FA-hydrolysates, on the

other hand, had no reducing capacity. It is possible that the presence of lactate and formate in LA and FA-hydrolysates, respectively are responsible for interference with the FRAP assay. Marques et al. reported that buffer systems significantly influence results in a Cu(III)-reducing assay.[130]

Contrary to a previous report that reducing capacity remains unchanged with increase in DH,[7] a significant increase in reducing capacity was observed with the FL hydrolysate withdrawn at 6 hours (**Figure 3.8. B**). However, the reducing capacity of FL-hydrolysate decreases on further increase in DH, which can be due to the degradation of active peptides into inactive fragments and amino acids with extended enzymatic hydrolysis.

3.3.5.3. Sacrificial Antioxidant Activity (GSH protection)

Sacrificial antioxidant activity of peptides was assessed based on their ability to protect glutathione (GSH, responsible for the maintenance of redox status in most forms of life) from oxidation due to Fenton's reaction. Udenigwe et al. suggested that smaller sized peptides possess better ability to protect GSH *via* improved solvent and free-radical accessibility.[115] However, it was observed in this study that the salmon visceral hydrolysates with higher DH (**Figure 3.6. C**) had a lower capacity to protect GSH (**Figure 3.8. C**). DH and GSH protection had the following

correlation coefficients for LA, $\rho = -0.310$, $p = 0.423$, $n = 8$; FA, $\rho = -0.714$, $p = 0.037$, $n = 8$; and FL, $\rho = -1.000$, $p = 0.083$, $n = 4$. Although these relationships were not statistically significant, largely because of the smaller sample size, it was indicative of the negative impact, increase in DH (smaller peptides) has on the capacity of the hydrolysates to protect GSH.

Radical scavenging activity of salmon protein hydrolysates have been reported to be higher for increasingly hydrophobic hydrolysate fractions, with the exception of hydroxyl radicals.[42] For FA-hydrolysates, S_o was negatively correlated (ρ = -0.333, p = 0.387, n = 8) with GSH protection. In contrast, for LA and FL processed hydrolysates, increase in S_o led to a higher sacrificial antioxidant activity with positive correlation between S_o and GSH protection (LA, ρ = 0.643, p = 0.071, n = 8; and FL, ρ = 1.000, p = 0.083, n = 4).

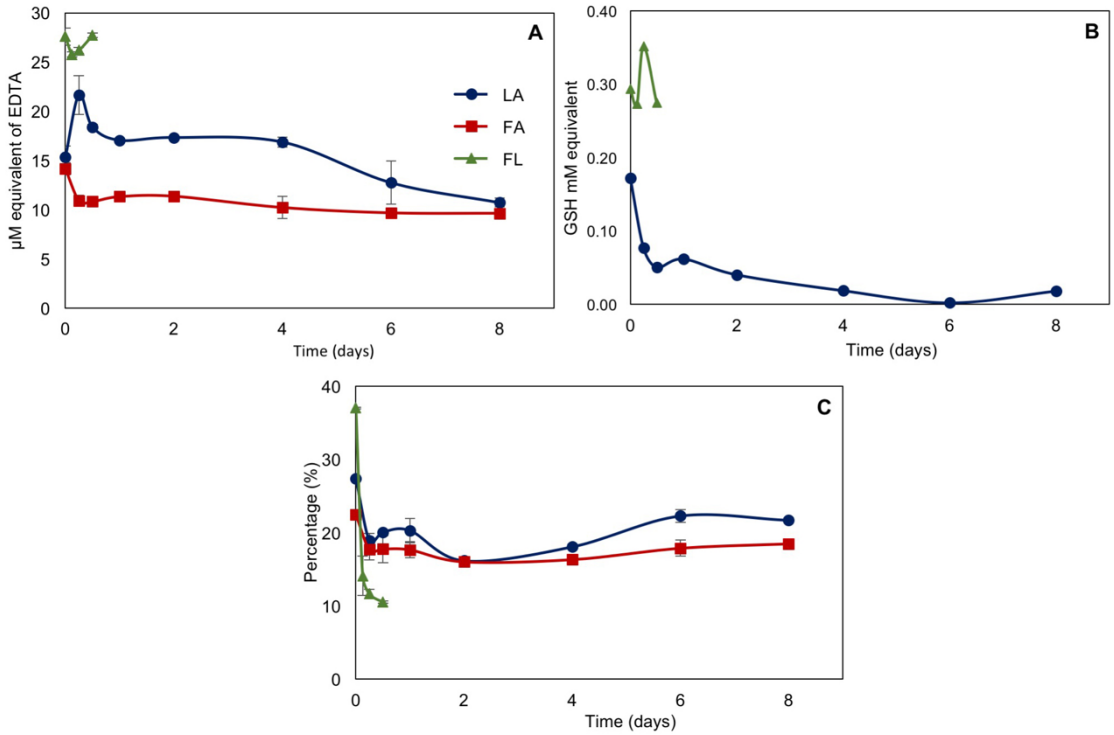

Figure 3. 6. Characterization of antioxidant activities of the salmon visceral hydrolysates prepared from LA, FA and FL. (A) Fe(II) chelation activity expressed as μM EDTA equivalent, (B) Ferric reducing capacity expressed as mM glutathione (GSH) equivalent, and (C) Sacrificial antioxidant capacity of the hydrolysates assessed and expressed as percentage

Even though it was expected that FL-hydrolysate would enable GSH protection *via* higher metal chelation capacity (making Fe^{2+} unavailable for facilitating Fenton's reaction), the results were contrasting to our prediction. Therefore, the exact mechanism of GSH protection remains unclear, although it is identified that larger peptides (hydrolysates withdrawn at initial

time points with lower DH) were observed to have a better ability to quench the hydroxyl free radicals formed as a result of Fenton's reaction and protect the GSH.

3.4. Conclusion

The present results indicate that the use of endogenous proteases is possible for the extraction of functional protein hydrolysates from salmon by-products. The pH and temperature are important parameters that determine the activity of a specific set of proteases within tissues. Commercial proteases such a Flavourzyme has its benefits in terms of a uniform cleavage pattern that maintains uniform functional characteristics, thereby contributing to enhanced antioxidant activity. A spectrum of varying properties was observed for the hydrolysates prepared from the same parent protein source. Therefore, this study highlights the significance of enzymatic cleavage specificity/pattern as compared to the cost, quality and properties of the protein source, in determining functionality and antioxidant functional properties of resultant peptides.

CHAPTER 4

Influence of proteolysis parameters on the recovery, physico-chemical and antioxidant properties of ground by-product protein hydrolysate derived from Atlantic salmon (*Salmo salar*)

Abstract

The influence of two autolytic approaches involving formic acid treatment (FA) and lactic acid fermentation (LA) on salmon total by-product mix (proportionate composition of head, skin, frame and viscera) was investigated (8 days) and compared to enzymatic proteolysis-processing using Flavourzyme (FL, fast processing, 12 hours). Bioprocessing approaches utilized inherent moisture in the ground fish tissues (no additional water). Proteolytic activity during bioprocessing varied significantly between all three approaches (LA<FA<FL at initial time points), while protease activity increased significantly for LA with time, it decreased with time in FL and FA application. Degree of hydrolysis in LA fermentation (8 days, 41.75 ± 0.98%) was on par with FL-hydrolysates (0.5 days, 46.61 ± 4.29%), while that of FA-autolysis was significantly lower (8 days, 35.22 ± 1.50%), although FA treatment showed the highest total soluble protein recovery (7.53 ± 2.64% of salmon by-product mix). FL-hydrolysates demonstrated the highest Fe(II) chelation (66.58±7.93%) and Fe(III) reducing capacity (221.4 ± 11.79 GSH μM equivalent), while both the autolytic approaches displayed higher sacrificial antioxidant activity (% protection of glutathione; LA, 8 days, 29.04 ± 4.06%; FA, 8 days, 26.92 ± 4.42%). The present study describes the potential of commercially viable protein hydrolysate recovery from salmon processing waste streams with potential applications in functional foods and food storage.

4.1. Introduction

Fish processing by-products are commonly recognized as low-value waste-streams with negligible market value. Additionally, disposal of these waste streams is both expensive and a major cause of environmental pollution.[3] Considering the large amounts of food-grade proteins in fish waste materials, proteolysis based processes are being developed to recover proteinaceous material from under-utilized fish and fish by-products that can potentially be converted into marketable and acceptable forms, which can be widely used in the food industry (nutraceuticals,[131] food ingredients,[132] functional foods[133] or natural preservative)[134] rather than as animal feed or fertilizer. Salmon processing wastes from typical filleting plants includes a mix of head, frame, viscera and skin.[2]

Functional properties of fish proteins can be improved by proteolysis under controlled conditions.[7] Several reports have been published on process optimization for valorization of salmon by-products, with the objective of improving protein recovery along with imparting desired functional and nutritional properties.[2,33,63,68,79,89,99,135] Enzymatic,[46] bacterial[68] and chemical[76] processing approaches have been utilized to enable hydrolysate recovery and improve bioactivities. Protein hydrolysis potentially influences the molecular size, hydrophobicity and polar groups of the hydrolysate.[7,39] The physicochemical characteristics of the protein hydrolysate directly affect the functional properties,[40] yield[54] and use.[136]

The objective of this study was to monitor and elucidate the role of processing approaches in preparing protein hydrolysates from Atlantic salmon co-products, with high protein recovery and marketable physicochemical and antioxidant properties. The present study was part of an ongoing research initiative to develop processes and products that valorize and

facilitate the full utilization of by-products from Atlantic salmon (*Salmo salar*), a major commercial fish species in Atlantic Canada and around the world.

4.2. Materials and Methods

4.2.1. Preparation of Slurry

Frozen by-products derived from market size Atlantic salmon (*Salmo salar*) was obtained from a local filleting plant (Cooke Aquaculture, PEI., Canada). These were thawed (in room temperature (RT)), weighed and blended (separately, without additional moisture) to form a thick semi-solid. Ground tissues (salmon head, frame, skin and viscera) were stored separately at -20 °C till further processing. For bioprocessing, 2800 g of the total by-product mix, comprising of head (500 g), frame (950 g), skin (700 g) and viscera (650 g) were withdrawn and added to a sealed batch fermentor (New Brunswick™ Bioflow®/CelliGen® 115, GMI Inc., MN, USA) and mixed to uniformity prior to the treatments. The salmon total by-product mix was prepared based on the yields of individual components (head, frame, viscera and skin) from the filleting plant.

4.2.2. Processing Approaches

4.2.2.1. Formic Acid (FA) Treatment

Homogenous by-product mix was subjected to treatment with formic acid (88%, Thermo Fisher Scientific, ON, Canada) aqueous solution (2%, w/w) (modified from Vieira et al., 2015).[108] The temperature of the slurry was maintained at 37 °C and agitated continuously at 150 rpm. Samples were withdrawn at different time points (0, 0.25, 0.5, 1, 2, 4, 6 and 8 days) and immediately stored at -20 °C till further analysis.

4.2.2.2. Lactic Acid (LA) Fermentation

To the salmon total by-products mix, deproteinized whey (5% (w/w) containing 80-90% lactose, obtained from Saputo Inc., Montreal, Canada) was added along with 1% lactic acid

bacteria (LAB, *Lactobacillus plantarum* CNCM MA 18/5U (>1 × 10^{10} cfu/g), *Pediococcus acidilactici* CNCM MA 18/5U (>1 × 10^{10} cfu/g)) inoculum (LALFEED® LACTO, Lallemand Inc., Aurillac, France) and were mixed thoroughly. Temperature and stirring conditions of the slurry were maintained as previously mentioned for FA treatment. Samples were withdrawn (0, 0.25, 0.5, 1, 2, 4, 6 and 8 days) and stored at -20 °C until further analysis.

4.2.2.3. Flavourzyme Proteolysis (FL)

Aspergillus oryzae protease preparation, Flavourzyme (Sigma-Aldrich, ON, Canada, EC. 232-752-2) was mixed (enzyme-to-substrate ratio of 1:100 (w/w)) with the slurry. pH was monitored and maintained automatically at 7.0 with 0.1 M NaOH, and enzymatic hydrolysis was carried out under the same temperature and stirring conditions as the FA and LA processing. Increasing FL proteolysis beyond the threshold of 12 hours led to fouling of the by-product mix as was in the case of salmon viscera. Samples were withdrawn (0, 3, 6 and 12 hours (0.0, 0.125, 0.25 and 0.5 days, respectively)) and stored at -20 °C.

4.2.3. Characterization of the Bioprocessing Approaches

4.2.3.1. Lactose/Lactic Acid Determination

Lactose/lactic acid content was determined using a Flexar high performance liquid chromatography (HPLC) system equipped with a refractive index detection (PerkinElmer, Shelton, CT, USA) from the frozen samples collected at different time intervals from LA fermentation (as described in section 3.2.3.2.).

4.2.3.2. Colony Count to Estimate LAB Growth

Serially diluted samples (1×10^{-7}) collected at different intervals were spread (100 µL) on DeMan-Rogosa-Sharpe (MRS) agar (Sigma-Aldrich, MO, USA) plates (incubated at 37 °C for 2

days) to estimate the changes in growth of LAB over the course of the LA fermentation. Results were expressed as colony forming units per mL (cfu/mL).

4.2.3.3. Protease Activity over Time

Protease activity within the ground salmon by-product mixture was estimated using hemoglobin as the substrate (Modified from Ichishima and Yoshida (1963)).[109] The assay protocol and calculations were identical to salmon visceral bioprocessing to calculate protease activity in units/mL (section 3.2.3.4.).

4.2.4. Compositional analysis of processed salmon by-product mix

4.2.4.1. Fractionation and Yield

Samples (8 g) collected at the respective time intervals mentioned earlier were transferred to Falcon™ 50 mL conical centrifuge tubes and centrifuged (3,500×g) for 15 min. The different fractions were then collected into weighed tubes and freeze dried. Both wet and dry weights were used to calculate the yield and moisture content.

4.2.4.2. Fluorometric Detection of Lipid Peroxides

Relative lipid peroxide content in the centrifugally separated protein hydrolysates was estimated with Diphenyl-1-pyrenylphosphine (DPPP, Molecular Probes™, OR, U.S.A.), a fluorescent probe to detect levels of lipid peroxidation (section 3.2.4.6.).

4.2.5. Determination of soluble protein content and total protein recovery

Soluble protein content of the lyophilized crude protein hydrolysate (obtained by centrifugal (3,500×g) separation) was estimated using the Pierce™ Modified Lowry Protein Assay Kit (ThermoScientific™, ON, Canada) according to the manufacturer's instructions for samples at 1 mg/mL concentration (in d.H_2O, As in section 3.2.4.2.).

The total protein recovered per 100 g of salmon by-product mix was calculated based on the formulae:

$$\text{Protein recovery (\%)} = \text{dry yield of hydrolysate fraction} \times \frac{\text{Soluble protein content (\%)}}{100}$$

where,

$$\boldsymbol{Dry\ yield\ of\ protein\ hydrolysate}\ (\%) = \boldsymbol{aqueous\ fraction\ yield}\ (\%) \times \left(\mathbf{1} - \frac{\boldsymbol{moisture\ content}\ (\%)}{\mathbf{100}}\right)$$

4.2.6. Characterization of the Hydrolysates

Salmon by-product protein hydrolysates were prepared by centrifuging (3,500×g) the slurry (collected at different time intervals mentioned above) for 15 min. The liquid hydrolysate fraction was collected separately, lyophilized and stored in -20 °C until further analysis.

4.2.6.1. Degree of Hydrolysis

Degree of hydrolysis (DH) was assessed and calculated based on the method reported by Nielsen et al., 2001[39,110], and has been previously described in section 3.2.4.1.

4.2.6.2. Determination of Surface Hydrophobicity (S_o)

Fluorometric determination of surface hydrophobicity was carried out with a hydrophobic probe, 8-Anilino-1-naphthalenesulphonic acid (ANS, Sigma-Aldrich, ON, Canada).[111] The slope of the fluorescence intensity vs. concentration plot was calculated to be the S_o value (same as 3.2.4.3.).

4.2.6.3. Free Thiol Determination

Sulfhydryl (SH) groups were determined (same as section 3.2.4.4.) and expressed as µM GSH equivalent using DTNB [5,5′-dithiobis-(2-nitrobenzoic acid), Sigma-Aldrich, WI, U.S.A.].[112]

4.2.7. Antioxidant Assays

4.2.7.1. Ferric Reducing Capacity

Ferric reducing capacity of the total by-product protein hydrolysates was carried out as in section 3.2.5.2. based on Mohan et al (2015).[39]

4.2.7.2. Metal Chelation

Metal (Fe(II)) chelation assay was performed with the help of FerroZine™ (5 mM, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p'*-disulfonic acid monosodium salt hydrate, Sigma-Aldrich, ON, Canada) based on Saidi et al. with modifications[114] (section 3.2.5.1).

4.2.7.3. Glutathione (GSH) Protection

The sacrificial antioxidant capacity of the hydrolysates was assessed using GSH protection assay, which measures the ability of the protein hydrolysates to protect GSH from Fe(II)/H2O2-induced oxidation (Fenton's reaction).[115] following the identical procedure as 3.2.5.3. The GSH protection capacity (%) of hydrolysate was calculated based on:

$$\frac{(\text{Abs of protected GSH (sample + GSH)} - \text{Abs of oxidized GSH})}{(\text{Abs of unoxidized GSH} - \text{Abs of oxidized GSH})} \times 100$$

4.2.8. Statistical Analysis

Triplicate analyses were performed and the results were expressed as mean ± std. deviation. Two-way analysis of variance (ANOVA) was performed followed by Tukey's HSD test for the separation of means using SAS® university edition (SAS Institute Inc., Cary, NC, USA) along with the macro, pdmix800.[116] Spearman's rank order coefficient (ρ) was calculated to determine correlation (no level of significance if $p>0.05$) between different variables using Sigmaplot 12.1 (Systat Software, San Jose, CA, USA).

4.3. Results and discussion

A FAO report on salmon by-products from Atlantic salmon in Chile quoted individual by-product yields to comprise approximately 23% (of total by-products) frame, 5% skin, 48% head and 24% viscera of the total by-products generated.[21] Whereas, Atlantic salmon harvested and processed from South Australia found yields to be ~26% frame, ~26% skin, ~18% head and ~30% viscera.[2] In contrast, our estimation based on weighed by-products were 34% frame, 25% skin, 18% Head and 23% viscera of the total by-product waste generated per salmon from the local filleting plant in PEI., Canada. The reported findings[21] were based on surveys with processing-plants and are subject to shortcomings based on the provided answers.

The total by-product mixture had lower protease content with the viscera and head fractions being the largest reservoirs of proteolytic enzymes.[137] As opposed to the immediate liquefaction, as was the case of salmon visceral processing, the total by-product mix took 1-2 hours for complete liquefaction in the case of all the bioprocesses. Additionally, it was observed that with the increase in processing time, the oil phase along with components of the skin separated on to the top of the fermenting/hydrolysate batch while the residual material composed mostly of bones (from the frame) settled at the bottom inside the fermentor.

4.3.1. Characterization of Bioprocessing

Unlike the FA application on processing of ground salmon viscera, it was observed that pH of FA treated salmon total by-product mix increased significantly with time from 3.36 (0 days) to 4.00 (8 days) (**Figure 4.1. A**). It has been demonstrated that formic acid decomposes at pH – 4, in the presence of Fe(III).[138] The increase in pH is possibly as a result of formic acid decomposition in salmon total by-product mix, which potentially has larger amounts of Fe as compared to just the viscera (lower Fe content has been demonstrated in beef viscera).[139]

As expected with LA fermentation, there was a significant dip in pH over time. However, the decrease in pH, was at a slower rate in total by-product (**Figure 4.1. A**) mix as compared with salmon viscera processing (**Figure 3.3. D**). This corroborates with the fluctuations seen in the composition of lactose (**Figure 4.1. B**), lactic acid (**Figure 4.1. C**) and LAB population **(Figure 4.1. D**) with time in LA fermentation.

Moreover, the growth of LAB was at a significantly slower rate as compared to visceral fermentation. Larger protein fragments (low DH) were present within salmon total by-product mix as compared to visceral processing resulting from lower levels of endogenous proteases, along with higher amounts of connective tissue (skin and frame). Moreover, smaller peptide size (higher DH) has been demonstrated to enable better LAB growth.[119] Therefore, the slower growth in total by-product fermentation was as a result of relatively lower efficiency of LAB in utilizing the substrate. Additionally, lactic acid production was higher (**Figure 4.1. C**) and the rate of decline of LAB population was at a much slower rate as compared to visceral processing (**Figure 3.3.**). Higher death rate of LAB populations has been attributed to higher concentration of NaCl and lower buffering capacity of substrate.[140] It is also noteworthy to mention that lactic acid production significantly increased after LAB growth ceased (1 day, **Figure 4.1. C**). This aligns with the kinetic model of LAB growth and decline proposed by Passos et al. (1994).[140]

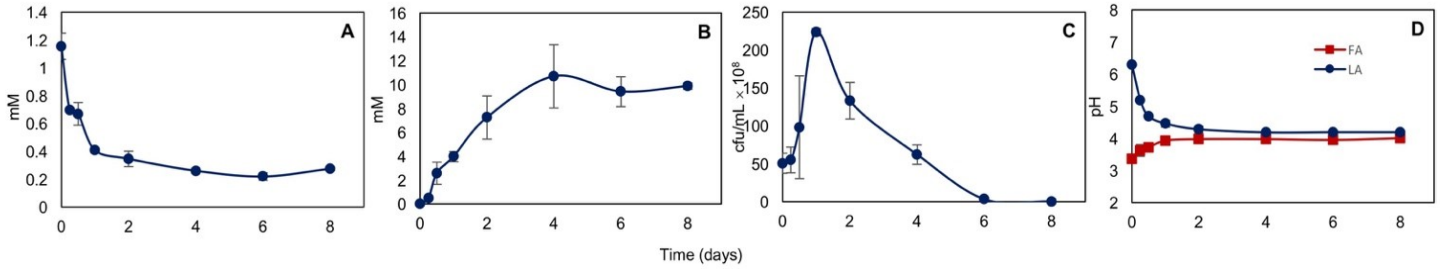

Figure 4. 1. Characterization of the lactic acid (LA) fermentation and formic acid (FA) treatment process: (A) Change in lactose concentration over time as quantified with HPLC. (B) Change in lactic acid concentration during fermentation. (C) Variations in the population of lactic acid bacteria (LAB) at different time points of LA fermentation as estimated through colony counts. (D) Change in pH during LA fermentation and FA treatment

4.3.2. Protease activity

Proteolytic activity in FL-proteolysis and FA treatment although initially higher, decreases significantly with time (**Figure 4.2.**). Protease activity in both approaches following a similar trend (although lower values for total by-product mix (lower levels of endogenous enzymes compared to viscera) over the course of processing as compared to visceral processing (**Figure 3.4.**). Proteolytic activity of proteases are known to decrease during the course of the hydrolysis, as a result of autodigestion,[95] and other matrix interactions.[123] In contrast, LA fermentation was slow in activation of proteases (**Figure 4.2.**). The change in pH in both autolysis approaches correlated negatively with the protease activity (LA, $\rho = -0.156$, $p = 0.662$, $n = 8$; FA, $\rho = -0.934$, $p = 2\times10^{-7}$, $n = 8$) i.e., the pH decrease of the ground by-product mix activates/liberates the proteases. Lysosomal catheptic proteases have been demonstrated to be primarily responsible for proteolytic activity (pH ~ 4) in pink salmon (*Oncorhynchus gorbuscha*) muscle tissues.[141,142] Another similar study has found cathepsin B-like enzymes to be most active at pH of around 3.0 in Pacific whiting.[143]

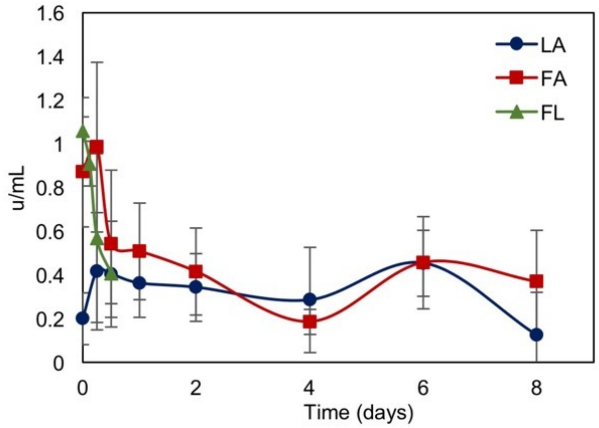

Figure 4. 2. Change in enzyme activity (U/mL) at different time points for the three different bioprocessing approaches; lactic acid (LA) fermentation; formic acid (FA) treatment; Flavourzyme (FL) hydrolysis.

4.3.3. Compositional analysis

Processed salmon total by-product mix withdrawn at different time points centrifugally separated (3,500×g) into four fractions (**Figure 3.1.**), namely (from bottom to top following centrifugation) residual (ash content and bone residue), hydrolysate (aqueous), sludge (lipids/emulsion (phospholipopeptidic complexes))[99] and oil fraction.

4.3.3.1. Centrifugal separation and yield of different fractions

The residual fraction decreased with time as the structural components in the salmon by-product tissues were hydrolyzed and degraded with time in all three processes (**Figure 4.1. A**). The negative correlation between yield of hydrolysate and residual content was consistent through all three approaches (LA, $\rho = -0.929$, $p = 2\times10^{-7}$, $n = 8$; FA, $\rho = -0.809$, $p = 0.009$, $n = 8$; and FL, $\rho = -1.000$, $p = 0.083$, $n = 4$). Formic acid treatment, being an established method to solubilize and extract proteins,[52] was superior in reducing residual fraction (**Figure 4.1. A**) and increasing the output of the hydrolysate fraction (**Figure 4.1. B**).

Similar to visceral FL-proteolysis, yield of sludge fraction was higher for FL application, whereas the sludge content in both autolytic approaches remained relatively low with no statistically significant difference during the course of processing (**Figure 4.1. C**). The oil yield did not change statistically with different approaches or with time (**Figure 4.1. D**). It is clear from the negative correlation between yield of the aqueous and oil fraction (not significant for FL hydrolysis; LA, $\rho = -0.810$, $p = 0.009$, $n = 8$; FA, $\rho = -0.929$, $p = 2\times10^{-7}$, $n = 8$; and FL, $\rho = -1.000$, $p = 0.083$, $n = 4$), that more and more peptides were binding to oil present in the slurry forming emulsion fraction.

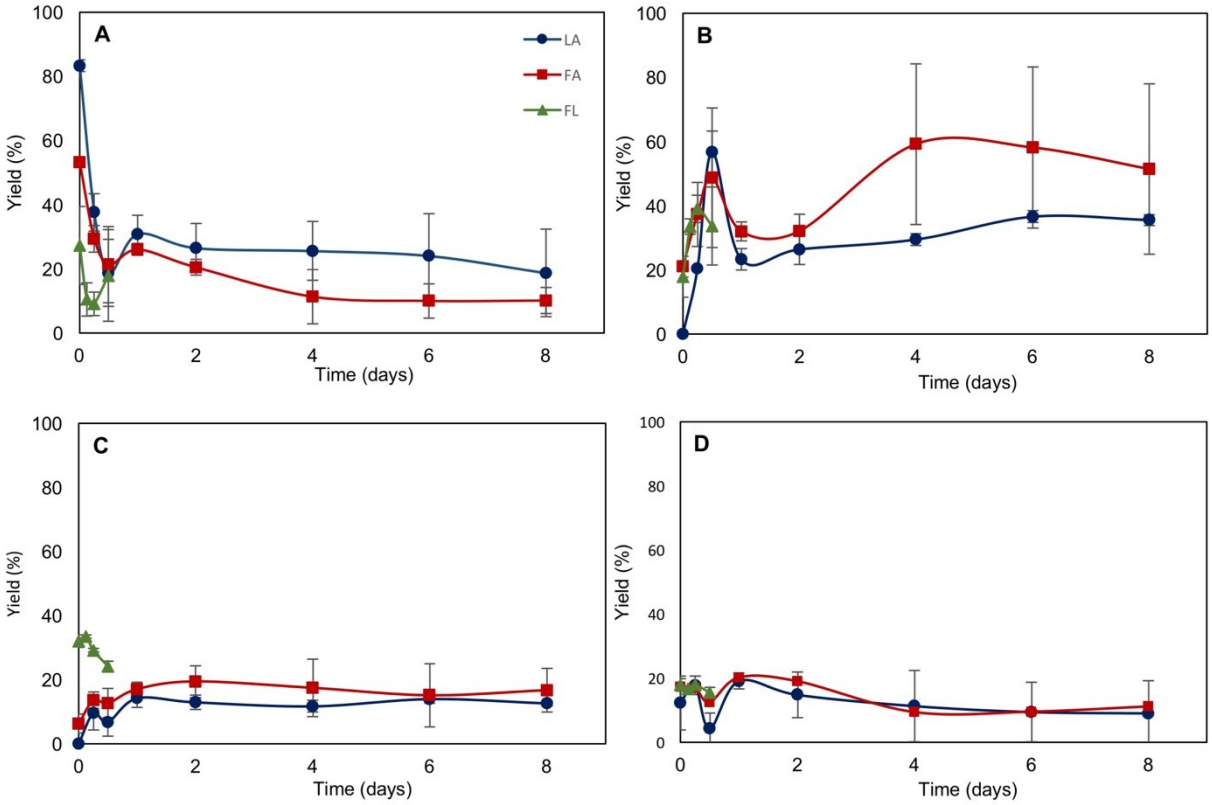

Figure 4. 3. Change in yield (wet weight basis) of the (A) Residue, (B) Hydrolysate, (C) Sludge, and (D) Oil over time for LA, FA and FL processing.

4.3.3.2. Moisture content of aqueous fraction

Addition of d.H_2O/moisture during the extraction or preparation phase of protein hydrolysates has been found to reduce marketable quality by imposing additional cost of water removal.[8] Similar to the solid state hydrolysis (Solid-state fermentation, SSF) approach described for viscera, the bioprocesses used here do not involve the addition of free water into the ground by-product mix or during the extraction of hydrolysate. In contrast, *Acanthogobius hasta* by-product SSF with *Aspergillus oryzae* was performed with addition of water to ensure 50% moisture content.[69] Extraction of fish protein hydrolysate is another step frequented with the addition of water.[46,69] Similar to visceral processing, moisture content of FA and FL was higher compared to LA (**Figure 4.4.**). Visceral protein hydrolysate fractions generated from all the three bioprocesses had higher moisture content than the respective hydrolysates from salmon total by-

product mix, which was expected as salmon viscera has been reported to have the higher moisture than other by-product components.[2]

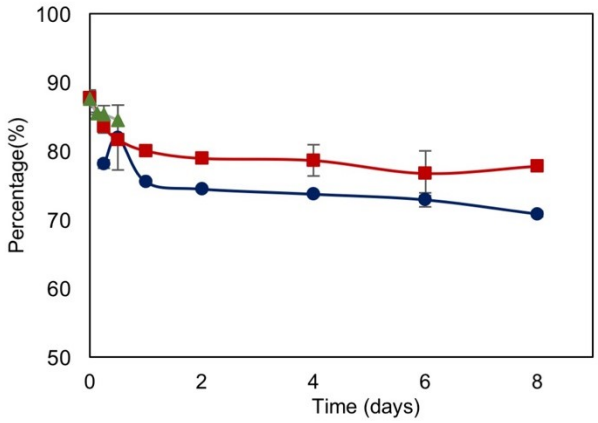

Figure 4. 4. Variation in moisture content of the aqueous fraction for LA, FA and FL processing.

4.3.3.3. Quantification of relative lipid peroxide content in aqueous fraction

Presence of lipid peroxides in food materials has been associated with organoleptic, nutritional and health-related concerns. Lipid peroxides have been associated with reduced shelf life of processed food-products and are recommended to be reduced as much as possible by recovering the oil fraction during preparation of the fish by-products. In contrast to the bioprocessing of salmon viscera, oil recovery from the total by-product mix did not significantly vary with time for each of the individual bioprocessing approach (**Figure 4.5.**). However, it has been established that salmon heads and skins are large reservoirs of polar lipids[144] (as opposed to triglycerides in the viscera), these components can emulsify fatty acids and other constituents of fish oil along with peptides generated from hydrolysis to form phospholipopeptidic complexes,[99] which can separate into the sludge fraction as well as in the hydrolysate fraction depending on the level of complexation. Thus, presence of lipid peroxides in the hydrolysate

fraction was predicted to be inversely related to yield of sludge fraction as emulsified/complexed oil accumulated into the sludge fraction. As expected, levels of lipid peroxidation negatively-correlated significantly with yield of sludge fraction when all the three bioprocessing approaches were compared together (ρ = -0.934, p = 3.2×10^{-4}, n = 19). Emulsifying ability of peptides generated has been widely demonstrated to decrease with DH,[96] the lipids in the phospholipopeptidic complexes (formed from peptides emulsifying the lipids) were expected to be released/ejected from the aqueous fraction with an increase in DH and the results indicated a negative correlation between lipid peroxide values and DH in all three approaches (ρ = -0.530, p = 0.019, n = 19). FL-proteolysis with significantly higher DH, was demonstrated to have the lowest amounts of lipid peroxide (**Figure 4.5.**).

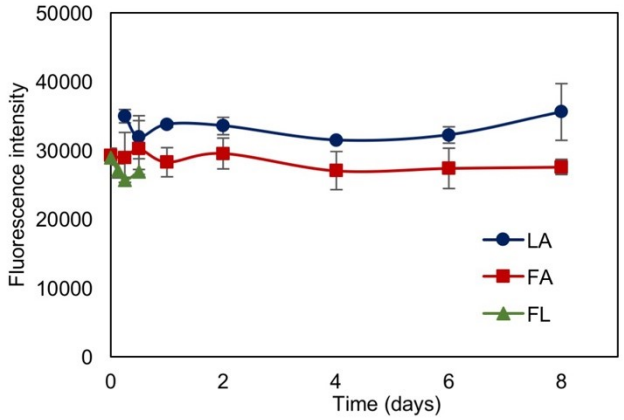

Figure 4. 5. Quantification of lipid peroxides present in the salmon total by-product protein hydrolysates withdrawn at different intervals measured as fluorescence intensity.

4.3.4. Total soluble protein recovery

The Lowry assay determines the total level of dissolved protein in solution. The salmon by-product mix contains larger proportion of insoluble structural proteins and was expected to have lower values when compared to processed salmon viscera (**Figure 3.6. B**). However, according to a previous report, Atlantic salmon head, skin and frame have a significantly higher

level of total protein content (as measured by BCA method).[2] The soluble protein content in visceral processing was higher for all three bioprocessing approaches at most of the time points (**Figure 3.6. B**) as compared to total by-product mix (**Figure 4.6. A**). Higher DH and enzymatic activity observed during processing of salmon viscera is expected to increase the soluble protein content in the aqueous fraction.

Properties of dilute formic acid as a protein solvent,[52] ensured higher values of protein solubility and recovery in total by-product protein FA-hydrolysates. In addition, Chinook salmon (*Oncorhynchus tshawytscha*) myofibrillar proteins have been demonstrated to be insoluble between pH<7.0 and >4.0,[145] this mechanism possibly underlies the decreasing trend in solubility (**Figure 4.6. A**), for both LA fermentation (pH decrease from 6.29 to 4.19) and FA treatment (pH increase from 3.36 to 4.00).

Total soluble protein recovery or protein recovery (%) is introduced as a broader term (in comparison to soluble protein content), encompassing both, soluble protein content and dry yield of the aqueous fraction. The total soluble protein recovery establishes the net mass of soluble protein extracted per 100 g of ground fish tissues. It was observed that protein recovery increased for all three bioprocesses with time (till 12 hours of FA treatment and LA fermentation, 6 hours for FL-proteolysis) and saturated (**Figure 4.6. B**). Moreover, in contrast to soluble protein content (%) data, the total soluble protein recovery was higher for FA and FL processing of total by-product mix (**Figure 4.6. B**) in comparison with FA and FL application in ground viscera (**Figure 4.6. B** (insert graph)), which is attributed to the higher inherent protein content of salmon head, skin and frame.[2] Contrastingly, LA fermentation of salmon viscera (higher amounts of endogenous enzymes (higher enzymatic activity as well as E/S ratio)), was more efficient at protein recovery than LA fermented total by-product mix fermentation (**Figure 4.6.**

B). Similar results were demonstrated in a previous report that focused on the effect of preparatory parameters such as initial protein content in the sample, temperature, pH and protease concentration during enzymatic hydrolysis on the recovery of protein content from veal bone hydrolysates,[41] which found temperature (40-60 °C) and pH (5.5-7.5) to be statistically insignificant, while influence of duration of hydrolysis and enzymatic concentration (E/S ratio) were important.[41] The protein recovery from LA-fermentation is on par with FL-proteolysis even though FL processing had higher protease activity. Similarly, type of protease (Flavourzyme, Neutrase and Alcalase) has been demonstrated to have no influence on protein yield from Atlantic salmon by-products (heads and frames), which was found to be dependent on E/S ratio, DH and processing time.[89]

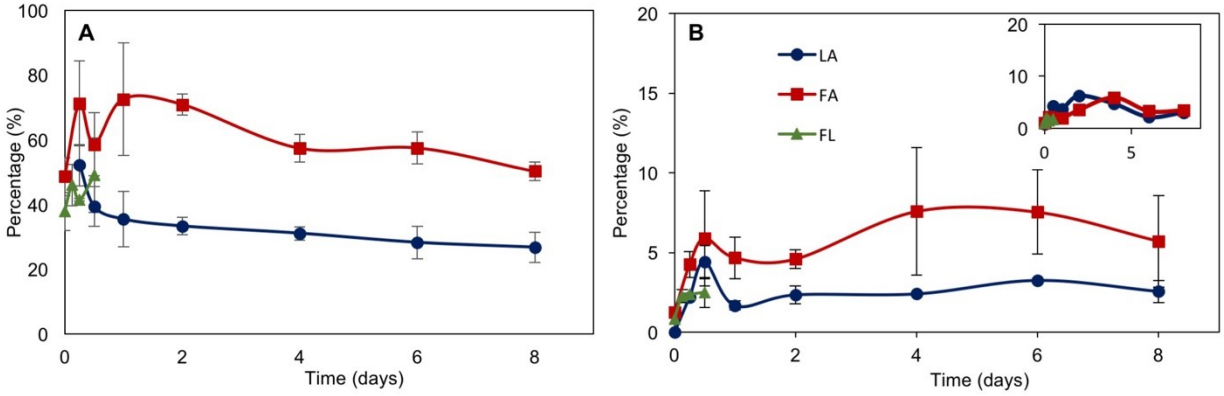

Figure 4. 6. (A) Soluble protein content (%) estimated through modified Lowry assay (B) Total soluble protein recovery.

4.3.5. Physicochemical characterization of crude protein hydrolysates

By visual inspection, the crude protein hydrolysate prepared/suspended in d.H_2O had a slight yellow color similar to salmon visceral hydrolysate. Higher Maillard reaction product (MRP) generation is associated with dark colored protein hydrolysate as in the case of the

brownish hydrolysate obtained *via* fermentation with *Bacillus subtilis*.[43] The processing approaches did not involve high heat application and would have limited generation of MRPs.

4.3.5.1. Degree of hydrolysis

Protein hydrolysates at high degree of hydrolysis result in improved solubility which has been associated with increased functionality.[146] However, above optimal high degree of hydrolysis can also have enormously negative effects on the functional properties.[136] As expected, the commercial protease (FL) had induced highest DH (0.25 days, 49.46 ± 1.38%). Even at initial time points (0 hour), FL hydrolysis had significantly higher DH than LA fermentation and FA treatment (**Figure 4.7. A**). The unhydrolyzed structural proteins in the by-product mix at time point 0 hour was predicted to be insoluble, remaining in the residual fraction following centrifugation. The FL aqueous fraction recovery was low at initial time point, however, the DH of recovered aqueous fraction was high as a result of the presence of peptides released from rapid enzymatic hydrolysis. LA-fermentation in contrast, initiates hydrolysis at a slower rate, with zero recovery of aqueous fraction at initial time point (0 hours). DH of LA-hydrolysates at final time point (8 days, 41.75 ± 0.98%) was however, on par with FL-hydrolysate (46.61 ± 4.29% (0.5 days, **Figure 4.7. A**). Similarly, Atlantic salmon pyloric caeca enzyme extracts has been shown to be comparable to commercial alkaline proteases (Alcalase, Flavourzyme, Corolase) on DH of protein hydrolysates.[47] In contrast to salmon visceral fermentation, DH of LA-fermentation over time is significantly higher than FA treatment. The endogenous enzymes over a wide pH spectrum along with microbial proteases generated during LA fermentation was predicted to contribute towards DH increase. Declining population of LAB (**Figure 4.1. C**), which has been demonstrated to release microbial proteolytic enzymes,[44] is predicted to be underlying the significant increase in DH of LA-fermentation at 6 days (**Figure**

4.7. A). DH for FL hydrolysis did not vary significantly with time after 3 hours, as has been reported previously for protein hydrolysates prepared with Flavourzyme (DH remained constant or proteolysis saturated after 120 mins).[88]

4.3.5.2. Surface Hydrophobicity

8-anilino-1-naphthalenesulfonic acid (ANS) is mostly assumed to be a hydrophobic probe binding to protein *via* the organic moiety (anilino-1-naphthalene). ANS is also believed to strongly bind cationic groups of proteins through ion pair formation and is reinforced by the naphthalene moiety. Gasymov and Glasgow, (2007) studied ANS binding through fluorescence and circular dichroism spectroscopies,[147] concluded that enhanced fluorescence with a hypsochromic shift resulted from the interaction of the sulfonate group of ANS with the positively charged groups that reduced the intermolecular charge transfer (CT) rate constant of ANS.[147]

The results from our study indicated that FA-hydrolysate had significantly higher surface hydrophobicity (**Figure 4.7. B**). A similar increase was also observed for FA treatment of salmon viscera (**Figure 3.6. C**). Effectiveness of intermolecular charge transfer (CT) rate constant of ANS in water is as a result of fast reorientation of water molecules in the vicinity of ANS molecules. Decreased molecular reorientation dynamics associated with formic acid molecules was predicted to have decreased the CT rate, thereby increasing fluorescence in an environment with formic acid molecules. A previous report on the effect of pH of protein solution on ANS fluorescence,[148] demonstrated that lower pH (3.0) induced higher fluorescence as a result of electrostatic interactions with the anionic probe. However, S_o of LA-hydrolysates was low (**Figure 4.7. B, Figure 3.6. C**), even though LA-fermentate had comparably lower pH than FA-treated by-product mix at final time points. Therefore, dynamics of molecular

reorganization of molecules is suggested to be more important than pH in determining the surface hydrophobicity using ANS.

With significantly higher DH, LA-hydrolysates and FL-hydrolysates were possibly hydrolyzed into small peptides or free amino acids that prevented structural conformations that facilitated ANS interactions. Similar exogenous application of Flavourzyme has been demonstrated to decrease S_o with increased proteolysis.[115] Therefore, surface hydrophobicity in a complex protein-processing system is dependent on presence of chemical moieties, along with inherent properties of substrate proteins, length of generated peptides, intermolecular peptide interactions, and cleavage pattern of protease.

4.3.5.3. Thiol content

Free sulfhydryl (SH) content has been implicated with protein recovery as higher levels of disulfide linkages impact aggregate formation and rheological properties of the proteins.[54] The free sulfhydryl content correlated significantly with the soluble protein content of the hydrolysates for all three bioprocessing approaches ($\rho = 0.519$, $p = 0.022$, $n = 19$). Neutral and alkali pH ranges have been demonstrated to enhance formation of disulfide linkages in fish proteins in comparison with acidic pH, which increase the free thiol content.[54] The total by-product mix from salmon was observed to have a larger reservoir of protein sulfhydryl (9.75 – 28.64 GSH mM equivalent; **Figure 4.7. C**) as compared to the salmon viscera (1.51 - 5.76 GSH mM equivalent; **Figure 3.6. D**). Protein hydrolysis in FL and LA led to decrease in SH contents while FA application increased (till 0.5 days) and then decreased the free thiol content (**Figure 4.7. C**). Similarly, proteolysis has been shown to increase[125] and decrease[39] SH contents. Protein thiols mediate redox capacity of cells in living systems *via* electron donation to free radicals and

have been associated with antioxidant properties.[149] The influence of SH content of the protein hydrolysate on ferric reducing capacity has been discussed in the latter sections.

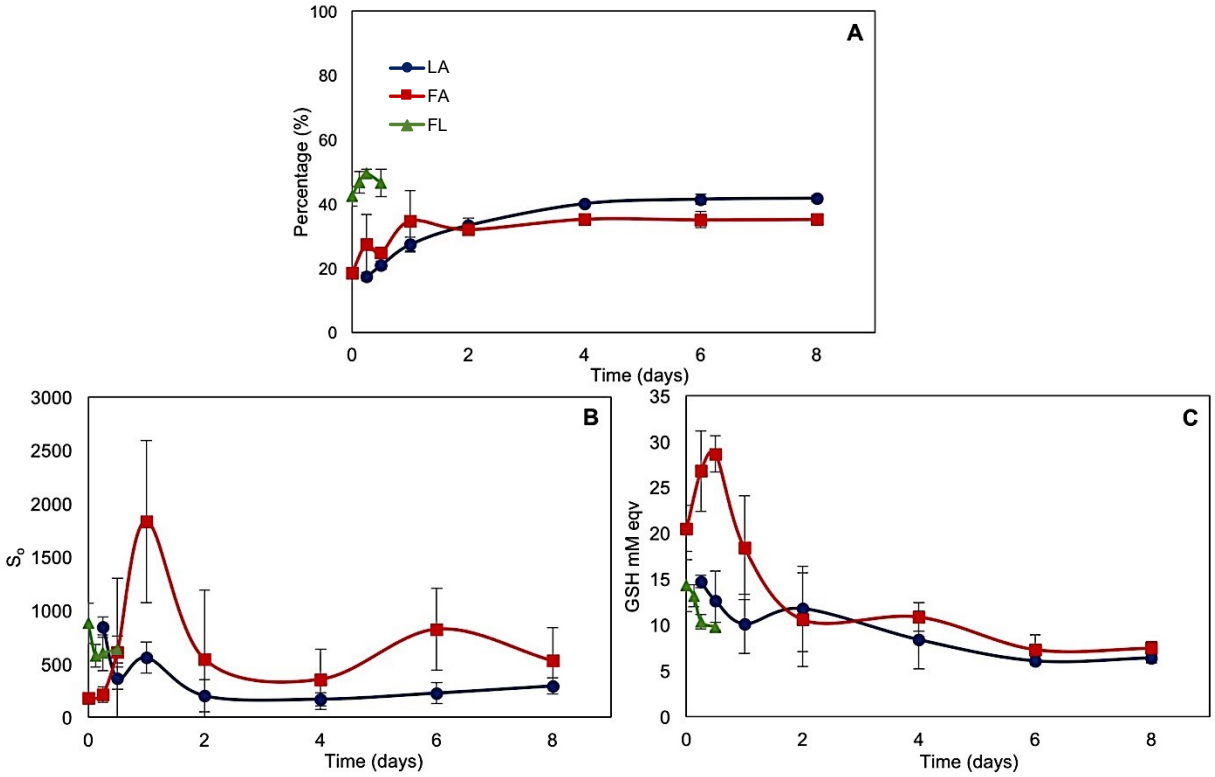

Figure 4. 7. Physiochemical characterization of the protein hydrolysate collected at different time points for FA, LA and FL: (A) Degree of hydrolysis (DH, %), (B) Surface hydrophobicity (S_o), and (C) Sulfhydryl concentration expressed as µM glutathione (GSH) equivalent.

4.3.8. Antioxidant properties of crude hydrolysates

4.3.8.1. Ferric reducing capacity

As mentioned earlier, the redox active SH content of the hydrolysate has been implicated with antioxidant properties and previous reports have suggested a relationship of SH content with ferric reducing capacity of protein hydrolysates.[39] In line with the predictions, ferric reducing capacity of the protein hydrolysates correlated positively with the sulfhydryl content (LA, $\rho = 0.810$, $p = 0.009$, $n = 7$; FA, $\rho = 0.619$, $p = 0.086$, $n = 8$; and FL, $\rho = 0.800$, $p = 0.333$, n

= 4). However, while SH content of FL-hydrolysates was relatively low (**Figure 4.7. C**), the ferric reducing potential of these peptides were significantly higher (**Figure 4.8. A**). Additionally, the reducing capacity of salmon visceral FL-hydrolysate was higher (**Figure 3.8. B**) as compared to FL-hydrolysate from total by-product mix (**Figure 4.8. A**), even though the latter was observed to have higher thiol content (**Figure 4.7. C**). Similar results were also observed in the case of LA- hydrolysate withdrawn at initial time points of both visceral and total by-products, visceral fermentates demonstrating higher reducing capacity. These mechanistic differences underlying the variation in ferric reducing capacity of salmon protein hydrolysates in relation to SH content can be attributed to the other contributing factors of peptide reducing capacity. For example, contrasting DH levels between protein hydrolysates generated from salmon viscera and total by-products. In fact, DH has been demonstrated to significantly influence the reducing capacity of protein hydrolysates.[150] Visceral hydrolysates had higher DH (**Figure 3.6. A)** as compared to total by-product protein hydrolysates (**Figure 4.7. A**) for all three bioprocesses.

As mentioned earlier, being a complex bioprocessing system, the chemical moieties (lactic acid, formic acid) present in the hydrolysate fraction can result in variations in the ferric reducing capacity assay.[130] However, impact of formic acid was limited in total by-product FA-hydrolysates, possibly as a result of the formic acid decomposition in salmon total by-product mix,[138] discussed in the section 4.3.1.

4.3.8.2. Metal (Fe (II)) chelation

Metal chelation capacity was almost non-existent for LA fermentation and FA fermentation with no significant increase observed for either of the processing approach (**Figure 4.8. B**). In contrast, FL-hydrolysis of by-product mix (as was in the case Flavourzyme addition to

salmon viscera) demonstrated a significant capacity to enhance the metal Fe(II) binding, with chelation capacity increasing significantly with time (**Figure 4.8. B**). At final hydrolysate concentration of 1.33 mg/mL (w/v), FL-hydrolysate withdrawn at 12 hours bound 66.58±7.93% of the added Fe(II) (24.52±1.93 EDTA μM equivalent). Generally, Flavourzyme has been shown to generate hydrolysates with high metal chelation capacity and the results obtained here were comparable with previous studies using minced fish muscle slurries (1:4 d.H_2O; 80-90% Fe(II) binding)[7] and flaxseed protein isolate (71.5%).[128]

FL-proteolysis of salmon visceral proteins, demonstrated higher Fe(II) chelation activity (~80%, **Figure 3.8. B**), however the visceral FL-hydrolysates showed a decrease metal chelation activity following a very high increase in DH (above optimal, **Figure 3.6. A**). Chelating capacity of salmon total by-product protein hydrolysate (lower DH (49%) as compared to salmon viscera (59%)) increased from 26.24±9.08% (14.70±2.21 EDTA μM equivalent.) to 66.58±7.93% (24.52±1.93 EDTA μM equivalent) with increase in DH (**Figure 4.8. B**). Similar results of increased metal chelation have been shown to be accompanying higher DH, during enzymatic hydrolysis.[7] Even though smaller peptides have tended to show higher Fe(II) chelation activity,[7] it is evident from the salmon viscera and total by-product protein hydrolysates that beyond an optimal point, the size of peptides cannot enhance metal chelation capacity.

Exogenous application of Flavourzyme has imparted Fe(II) chelation activity to peptides prepared from several different sources of proteins such as Flaxseed (protein concentrate),[128] fish muscle slurry,[7] crude ground viscera and ground by-product mix. However, FA treatment and LA fermentation did not alter metal chelation capacities of the salmon by-product protein hydrolysates (**Figure 4.8. B**). Similarly, peptic treatment resulted in moderate or negligible metal

chelating activities of half-fin anchovy (*Setipinna taty*).[87] The results outline the importance of cleavage pattern of proteases in determining the metal binding capacity of protein hydrolysates.

4.3.8.3. Glutathione protection

Radical scavenging/sacrificial antioxidant capacity of peptides were assessed based on their ability to protect GSH, which has been established to be responsible for maintenance of redox status in cells. In the assay conditions, decomposition of H_2O_2 was facilitated *via* Fenton's reaction in the presence of Fe(II) that leads to ·OH generation, which oxidizes GSH to GSSG, as GSH served as an electron donor.[115] Oxidized GSH (sulfhydryl) levels was determined using the dye DTNB. Molecular weight/size,[115] hydrophobicity,[42] and amino acid composition[151] have all been demonstrated to play a major role in determining the radical scavenging activity of peptides. Enhanced solvent and free-radical accessibility associated with smaller sized peptides have been proposed to underlie improved protection of GSH.[115] Moreover, radical scavenging activity of protein hydrolysates have been reported to be higher for increasingly hydrophobic hydrolysate fractions, with the exception of hydroxyl radicals.[42] Peptides have also been proposed to induce an antioxidant effect by chelation of pro-oxidant metal ions that catalyze the formation of free radicals.[151]

Even though FL-proteolysis demonstrated the higher DH (therefore smaller size), along with highest Fe(II) chelation capacity, the GSH protection capacity of FL-hydrolysates was the lowest (**Figure 4.8. C**). Similarly, salmon visceral hydrolysates (with higher DH) had a lower capacity to protect GSH from hydroxyl radicals (**Figure 3.8. C**). Flavourzyme generated peptides have been previously demonstrated to be low in radical scavenging capacity as compared to peptides prepared with Alcalase, Neutrase, Protamex, papain, and ficin from egg white powder.[152] The substantial exopeptidase activity of Flavourzyme compared to other

proteolysis approaches,[153] possibly resulted in generation of free amino acids that were not as efficient as intact peptides in exhibiting sacrificial antioxidant activity.

For LA-hydrolysates, the GSH protection capacity increased significantly with processing time (**Figure 4.8. C**). The hydroxyl scavenging activity of these protein hydrolysates as shown by the higher levels of GSH protection, correlated significantly with the DH (ρ = 1.000, p = 2.0×10^{-7}, n = 8) as well as the production of lactic acid (ρ = 0.929, p = 2.0×10^{-7}, n = 8) during processing. Moreover, lactate has been demonstrated to exhibit free radical scavenging activity in vitro,[154] and is being regularly used in the meat industry to preserve texture and color while preventing oxidation and lipid peroxidation.[155,156]

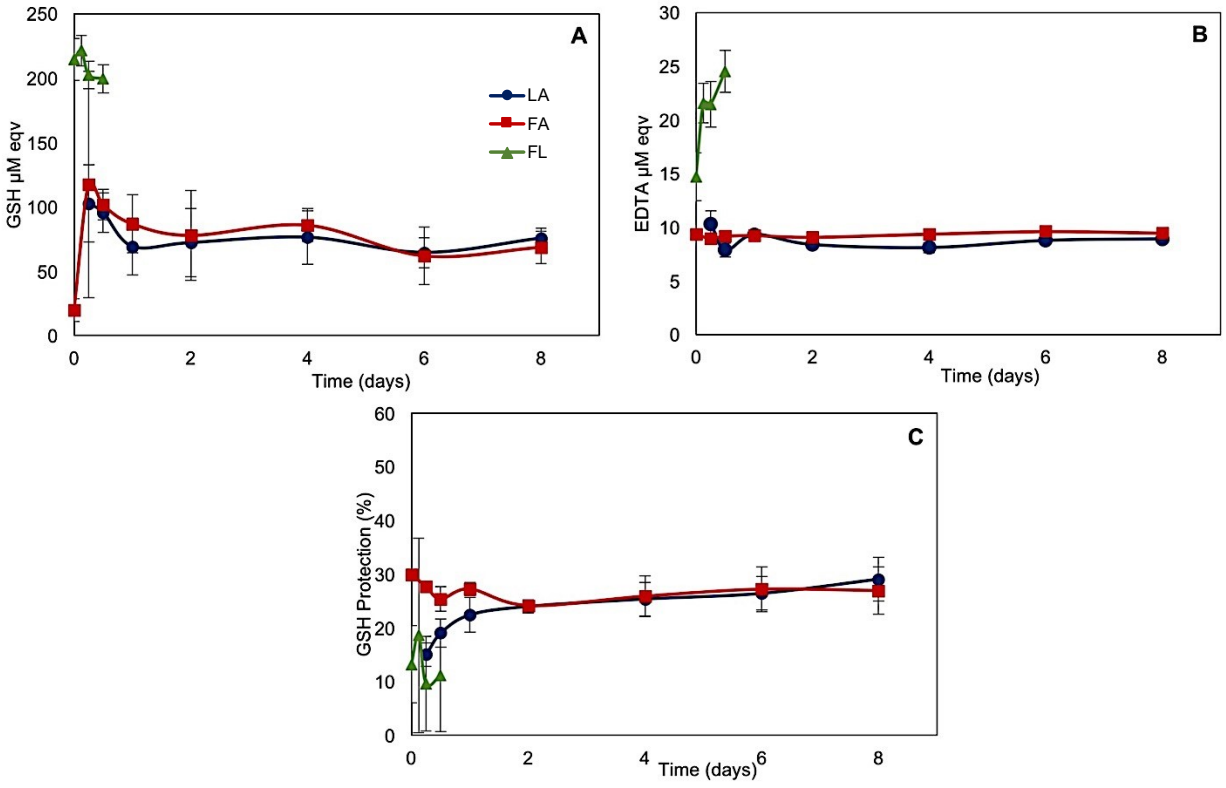

Figure 4. 8. Characterization of antioxidant properties of the salmon by-product protein hydrolysates prepared from LA, FA and FL. (A) Ferric reducing capacity expressed as mM glutathione (GSH) equivalent, (B) Fe(II) chelation activity expressed as µM EDTA equivalent and (C) Sacrificial antioxidant capacity of the hydrolysates assessed and expressed as percentage protection of GSH subjected to Fenton's reaction.

FA-hydrolysates on the other hand, had high sacrificial antioxidant activity that did not change significantly with time (**Figure 4.8. C**). The high sacrificial antioxidant properties can be attributed to peptides, generated early as a result of hydrolysis proceeding at a fast rate even at the initial time points. However, residual formate that may have remained in the hydrolysate fraction has the potential to react with the added ferrous chloride (source of Fe(II) for the Fenton's reaction) reducing its capability of generating hydroxyl free radicals.

From the results, it is proposed that several parameters are underlying the GSH protection capacity of proteinaceous materials, and includes cleavage specificity of processing protease, chemical moieties present in the complex mixture of protein hydrolysates, amino acid composition (free amino acids and peptide sequences), hydrophobicity and molecular weight (DH).

4.4. Conclusion

This study compares different preparation approaches on recovery and functionality of Salmon by-product protein hydrolysates. Commercially available lactic acid bacteria inoculum, formic acid and Flavourzyme enzymatic preparations were used in the production stage, followed by a single centrifugal separation step for obtaining protein hydrolysate. Each approach was unique in the recovery or yield and each imparted significantly different functional properties to the protein hydrolysate. Although Flavourzyme application appeared to enhance general antioxidant properties (reducing power and metal chelation), lactic acid fermentation and formic acid treatment had enhanced sacrificial antioxidant properties. While degree of hydrolysis was highest for Flavourzyme proteolysis, LA fermentation was on par with respect to protein recovery, whereas processing with formic acid resulted in the highest protein recovery. Processes were carefully controlled or monitored and the hydrolysate was prepared with the intention of

commercial application. Further research will be required to elucidate nutritional, textural, and sensory changes associated with salmon by-product preparation and incorporation into food matrices.

CHAPTER 5

Antioxidant properties of high and low molecular weight fractions of salmon (*Salmo salar*) by-product protein hydrolysate in aqueous, plasma-matrix and intracellular models

Abstract

Previous studies have demonstrated that cleavage pattern and other processing parameters significantly influence the antioxidant properties *in vitro*. Additionally, protein hydrolysate antioxidant activities have largely been attributed to low molecular weight peptides. In the present study, antioxidant properties of Atlantic salmon (*Salmo salar*) by-product protein hydrolysate of high and low molecular weight fractions were assessed in aqueous bioassays and compared to antioxidant mechanisms underlying protective effects within bovine plasma-matrix and a cellular (HT29) model subjected to hydroxyl radical-mediated oxidative systems. The low molecular weight (L-MW) Flavourzyme derived hydrolysate fractions exerted the highest Fe(II) chelation capacity (86.50±1.50% (29.13±0.39 EDTA μM equivalent)), whereas the corresponding high molecular weight (H-MW) fractions demonstrated the highest aqueous Fe(III) reducing capacity (447.46±12.62 GSH μM equivalent), plasma sulfhydryl protection (150.47±12.56%) and protection against plasma lipid peroxidation (~80-100%). In contrast, the autolytic hydrolysates (derived from lactic acid fermentation and formic acid treatment) were more effective in aqueous glutathione protection (25.70±0.53%), plasma ferric reducing capacity (166.06±8.77 GSH μM equivalent) and reduced intracellular lipid peroxide levels in H_2O_2-stressed HT29 cells. Findings from this study thus, highlight the range of antioxidant potential of salmon protein hydrolysates derived from processing waste.

5.1. Introduction

Adequate regulation of reactive oxygen species (ROS) underlies the proper functioning of living systems. There are multiple levels of regulatory and modulatory systems in place (glutathione, ascorbic acid, catalase, glutathione peroxidase, superoxide dismutase) to check the levels of ROS in the human body, whose production is also essential for defense and several other aspects. Generation of large amounts of ROS triggers oxidation chain reactions which modifies important biomolecules such as nucleic acids, lipids and proteins and is strongly correlated with several disease conditions and disorders.[157] The intestine especially is continuously exposed to ROS endogenously generated from luminal contents as well as from daily ingested oxidant components of foods. Literature extensively covers the pivotal role of ROS in the physiology and pathology of the intestine as well as in the microbiota profile.[158–160] In addition, many disorders associated with the gastrointestinal (GI) tract, such as inflammatory bowel disease and colon cancer are a result of excessive ROS generation and alteration in the redox status of intestinal cells.[161]

Predominantly hydrophobic peptides with cationic residues have been demonstrated to translocate into the inner mitochondrial membrane and induce antioxidant activity within the intracellular system.[157] Bioactive peptides have been shown to freely pass through membrane systems with no evidence of vehicular localization.[157] Zou et al extensively reviewed the structural features of proteins and peptides that underlie *in vitro* antioxidant properties.[162] While several physicochemical characteristics have been elucidated and attributed to antioxidant properties of peptides, antioxidant mechanisms of food-derived peptides is not yet clearly understood especially at a physiological level and in complex matrices.

Differences in cleavage specificity during bioprocessing is expected to lead towards varying physiochemical properties (Free amino nitrogen content, surface hydrophobicity, peptide size) of salmon hydrolysate fractions of differing molecular weight, and these differences are predicted to impact their interaction with components of the matrix and with free radicals. The present study focuses on evaluating antioxidant capacity of these hydrolysate fractions using physiologically relevant antioxidant assays, under normal and oxidative stress conditions. Antioxidant properties of salmon by-product hydrolysates are compared in aqueous *in vitro* systems, within protective mechanisms in a bovine plasma matrix and in an intracellular (HT29) environment model subjected to H_2O_2 stress.

5.2. Materials and Methods

5.2.1. Preparation of salmon by-product protein hydrolysates

By-products of market size Atlantic salmon (*Salmo salar*) were procured from a local gutting plant (harvested fish processed to HOG (Head on Gutted), and filleted off cut), where it was immediately frozen after processing. The salmon by-products were thawed, mixed and blended to form a representative mix (total salmon by product mix (section 4.2.1.) or simple visceral mix (section 3.2.2.)). The salmon by-product mix/viscera was processed using Lactic acid fermentation (LA), formic acid treatment (FA) and Flavourzyme proteolysis (FL) as described in sections 3.2.2. and 4.2.2. in sealed batch fermentors (New Brunswick™ Bioflow®/CelliGen® 115, GMI Inc., MN, USA). Crude salmon protein hydrolysates were prepared by centrifuging (3,500×g) the processed mix (collected at different time intervals) for 15 min. The liquid hydrolysate fraction was collected separately and freeze dried.

5.2.2. Molecular weight fractionation

The samples with prospective antioxidant capacity were chosen (**Table 1**) from each of the processing approaches based on preliminary studies (Chapter 3 & 4). The selected samples were subjected to ultrafiltration with a 3 kDa molecular weight cut-off ultrafiltration membrane using a tangential flow system (Pall Corp., ON, Canada). The retentate and filtrate were collected separately and lyophilized to obtain >3 kDa (Higher molecular weight (H-MW)) and <3 kDa (Low molecular weight (L-MW)) hydrolysate fractions, respectively.

Table 1. List of samples selected for molecular weight based fractionation and analysis

Tissue	Processing approach	Processing time (days)	Assigned sample name
Total	FL proteolysis	0.125	T1
Total	FL proteolysis	0.5	T2
Total	LA fermentation	0.25	T3
Total	LA fermentation	4	T4
Total	FA treatment	0.5	T5
Viscera	FL proteolysis	0.25	V1
Viscera	LA fermentation	0.25	V2
Viscera	LA fermentation	4	V3
Viscera	FA treatment	0.25	V4

5.2.3. Free amino determination

Free amino nitrogen (FAN) content of the different molecular weight fractions was calculated based on the method reported by Nielsen et al., 2001.[39,110]

5.2.4. Determination of Surface Hydrophobicity (S_o)

The hydrophobic probe, 8-Anilino-1-naphthalenesulphonic acid (ANS, Sigma-Aldrich, ON, Canada) was used in the determination of S_o of the protein hydrolysate fractions as described in section 3.2.4.3.[111]

5.2.5. Free Thiol Determination

Free sulfhydryl (SH) groups were determined using DTNB [5,5′-dithiobis-(2-nitrobenzoic acid), Sigma-Aldrich, WI, U.S.A.] as described in section 3.2.4.4.

5.2.6. In vitro antioxidant assays

5.2.6.1. Ferric Reducing Antioxidant Potential (FRAP)

Ferric reducing capacity of the hydrolysate fractions were carried out based on section 3.2.5.2 and expressed as GSH μM equivalent.

5.2.6.2. Metal Chelation

In vitro metal (Fe(II)) chelation activity of the salmon protein hydrolysate fractions were assessed as per section 3.2.5.1.

5.2.6.3. Glutathione (GSH) Protection

Sacrificial antioxidant capacity of the hydrolysate fraction was assessed using GSH protection assay (as per section 4.2.7.3.).

5.2.6.4. Antioxidant properties of Plasma Oxidation

Equal volumes of bovine plasma (at final concentration of 2.5 mg mL^{-1}, Sigma Aldrich, ON, Canada) and sample (final concentration of 1 mg mL^{-1}), were prepared in phosphate buffer saline (50 mM, pH 7.5) and pipetted into microfuge tubes which were mixed gently. H_2O_2 was added to the mixture at a final concentration of 50 μM (Fe is not added to induce Fenton's reaction, as Fe is already present in the plasma mix). The mixture was gently mixed and

incubated at 37°C for 2 hours. Oxidized (with H_2O_2) and unoxidized (without H_2O_2) plasma were also prepared similarly without test samples, as positive and negative controls

5.2.6.5. FRAP of oxidized Plasma

Protected/oxidized/unoxidized plasma was transferred (100 µL) into a new microfuge tube and 100 µL of potassium ferricyanide (1%) was added. The mixture was incubated at 50°C for 20 minutes and 100 µL of 10% TCA was added. The mixture was vortexed and 100 µL of the same was transferred into a 96 well plate, to which, 80 µL of deionized water and 20 µL of 0.1% $FeCl_3$ were added. These were then incubated at room temperature for 10 minutes and the absorbance was taken at 700 nm.

5.2.6.6. Plasma sulfhydryl (SH) protection

Protected/oxidized/unoxidized plasma (250 µL) was transferred into a new microfuge tube and 250 µL of TrisGlycine/SDS buffer (pH 8.3, 0.1M) was added. 5 µL of 5,5′-dithiobis(2-nitrobenzoic acid) (4 mg mL^{-1} in Tris-glycine buffer) was mixed with the sample and buffer solution. The mixture was incubated at 40°C for 15 minutes and 200 µL of the experimental mixture was transferred into 96 well plate and the absorbance was measured at 412 nm. The protection of plasma SH (%) was calculated:

$$\frac{(\text{Abs of protected plasma (sample + plasma)} - \text{Abs of oxidized plasma})}{(\text{Abs of unoxidized plasma} - \text{Abs of oxidized plasma})} \times 100$$

5.2.6.7. Plasma GSH levels

Protected/oxidized/unoxidized plasma was transferred (100 µL) into 96 well plate and 100 µL of 40 µM monobromobimane (mBBr) was mixed with the sample. The fluorescence was measured at excitation and emission wavelengths of 360 nm and 465 nm, respectively. Change in plasma GSH levels (%) as relative to oxidized plasma, was calculated based on the equation:

$$\frac{(\text{fluorescence of protected plasma (sample + plasma)} - \text{fluorescence of oxidized plasma})}{(\text{fluorescence of oxidized plasma})} \times 100$$

5.2.6.8. Protection from plasma lipid peroxidation

Diphenyl-1-pyrenylphosphine (DPPP, Molecular Probes™, OR, U.S.A.) was used as a fluorescent probe to detect levels of lipid peroxides in the bovine plasma treated with different hydrolysate fractions. DPPP oxide generated as a result of DPPP reacting with lipid peroxides, was detected fluorometrically at excitation and emission wavelengths of 361 nm and 380 nm, respectively.[113] DPPP (50 µg/mL, 50 µL, dissolved in DMSO and diluted using d.H_2O) was added to 150 µL of protected/oxidized/unoxidized plasma. Protection from plasma lipid peroxidation (%) was calculated using the equation:

$$\frac{(\text{fluorescence of protected plasma (sample + plasma)} - \text{fluorescence of oxidized plasma})}{(\text{fluorescence of unoxidized plasma} - \text{fluorescence of oxidized plasma})} \times 100$$

5.2.7. Intracellular antioxidant capacity of salmon protein hydrolysate fractions

Human colorectal adenocarcinoma derived HT29 cells (provided by Dr. Xu Zhang, Verschuren Centre for Sustainability in Energy and the Environment) were used in the intracellular antioxidant activity studies. HT29 cells were plated at 10^4 cells/mL density in 96 well plates and incubated for 24 hours prior to treatment with sample.

5.2.7.1. Cytoprotection

Cytoprotective capacity of the hydrolysate fractions was assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT, Sigma Aldrich, ON, Canada) assay.[163] To the plated cells varying (final) concentrations of sample (0.0625 – 0.5 mg/mL (100 µL+ 100 µL of media)) was incubated for 4 hours. Following incubation, media was withdrawn

and cells were washed with Hanks' Balanced Salt solution (HBSS). H_2O_2 at final concentration of 200 μM (diluted in HBSS) was pipetted into the wells and incubated again for two hours. Cells were washed again and MTT (0.5 mg/mL, 100 μL) was added to the cells and incubated for 4 hours. Thereafter, supernatant was removed and DMSO (100 μL) was added for color development and absorbance was measured at 565 nm. Cytoprotection capacity (%) of the protein hydrolysate fractions was calculated using the following equation:

$$\frac{(\text{Abs of oxidized treated cells} - \text{Abs of oxidized untreated cells})}{(\text{Abs of unoxidized untreated cells} - \text{Abs of oxidized untreated cells})} \times 100$$

5.2.7.2. Intracellular GSH levels

Intracellular GSH levels were measured using monobromobimane (mBBr), following the treatment with hydrolysate fractions in H_2O_2-stressed cells and also in unstressed cells.[163] Effects of peptide fraction treatments on the GSH levels in H_2O_2 stressed cells was carried out by subjecting the cells to 50 μM H_2O_2 (2 hours) following initial peptide fraction treatment (0.25 – 0.5 mg/mL (100 μL+ 100 μL of media), 4 hours) and washes. mBBr (40 μM, 100 μL) was then added to the wells. Since mBBr has to be detected fluorimetrically, the cells were then detached by the addition of 0.25% trypsin solution and transferred to a 96-well fluorescence microplate and fluorescence was measured at excitation and emission wavelengths of 360 nm and 465 nm, respectively. Similarly, protein hydrolysate treated unstressed HT29 cells were also assessed.

5.2.7.3. Intracellular lipid peroxidation

Lipid peroxidation levels in hydrolysate-fraction-treated HT29 cells were determined with diphenyl-1-pyrenylphosphine (DPPP, Molecular Probes™, OR, U.S.A.).[163] Treated cells were washed and either oxidative-stressed induced (50 μM H_2O_2 (2 hour incubation)) or unstressed. DPPP (50 μg/mL) was added into the wells and mixed. The contents were then

transferred into a 96-well fluorescence plate and the formed DPPP oxide was measured by relative fluorescence intensity (excitation and emission wavelengths of 361 nm and 380 nm, respectively).

5.2.8. Statistical analysis

The analyses were performed in triplicate and the results were expressed as mean ± std. deviation. Two-way (effects of sample processing and molecular weight fractions) and three way (effects of concentration, molecular weight fractions and sample processing) analysis of variance (ANOVA) was performed followed by Tukey's HSD test for the separation of means using SAS® university edition (SAS Institute Inc., Cary, NC, USA) along with the macro, pdmix800.[116] Spearman's rank order coefficient (ρ) was calculated to determine correlation (no level of significance if $p>0.05$) between different variables using Sigmaplot 12.1 (Systat Software, San Jose, CA, USA).

5.3. Results and Discussion

5.3.1. Free amino nitrogen content

Generally, FAN content was observed to be significantly higher in L-MW fractions (**Figure 5.1. A**). However, autolytic processed total salmon by-product hydrolysate fractions (T3, T4, T5) demonstrated significantly lower FAN, especially with L-MW fractions. T3 and T5 had insignificant differences between L-MW and H-MW fractions, while T4 H-MW fractions had higher FAN than corresponding L-MW (**Figure 5.1. A**). Peptide sequences (amino acid composition) are complex and can be degraded (deamidation, oxidation, hydrolysis, and cyclization reactions) during processing and storage leading to the loss of antioxidant activity.[164] For example, high temperatures may alter peptide structures, and lead to chemical modifications such as glycosylation as a result of Maillard reaction which may result in loss of functionality or

in some cases, improved functionality (reductones with high antioxidant activity). The mechanism underlying relatively lower FAN in autolytic total by-product hydrolysate fractions is unclear, especially since FAN content in visceral autolytic hydrolysate fractions are high. FL proteolysis resulted in higher FAN in both L-MW and H-MW fractions. Flavourzyme was predicted to result in higher FAN amounts especially in L-MW fractions, as a result of the generation of free amino acids as a result of high exopeptidase activity.[164]

5.3.2. Surface hydrophobicity

It was evident that S_o was higher in L-MW fractions of samples prepared with longer (duration) processing (T2, T4, **Figure 5.1. B**). Whereas, H-MW fractions prepared with shorter duration of processing (withdrawn initially) demonstrated a higher S_o (T1, T3). In a similar report, pepsin was effective in hydrolyzing peptides into smaller fractions leading to the increased exposure of internal hydrophobic amino acids.[164] However, most visceral hydrolysate fractions (L-MW and H-MW), with significantly higher free amino nitrogen (FAN) content (smaller sized peptides and free amino acids) had lower S_o (**Figure 5.1. B**). The trend observed in this study was comparable to the earlier mentioned study of peptic hydrolysates, where further hydrolysis of the peptic hydrolysate by trypsin resulted in the generation of more free amino acids and led to the decline in surface hydrophobicity.[164] The evidence suggests that optimal proteolysis and FAN contents increases the S_o, while very high FAN results in the lowering of S_o.

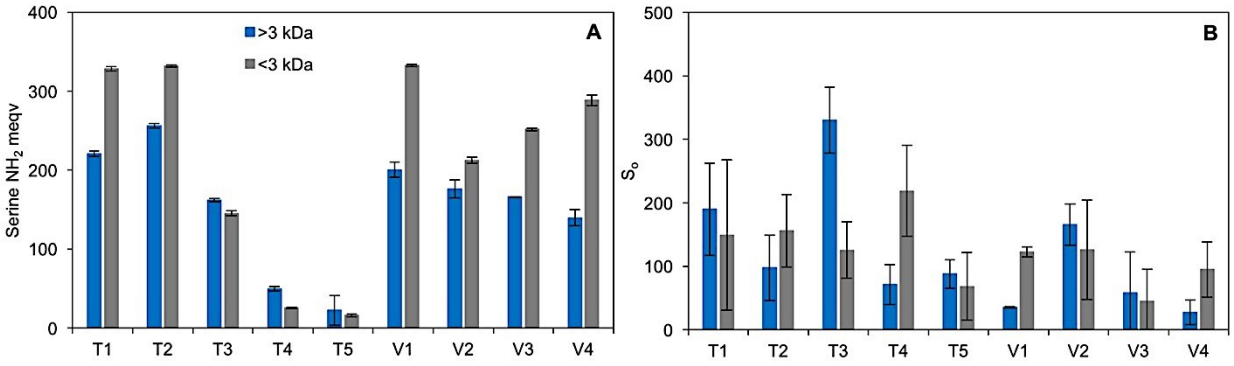

Figure 5. 1. Physiochemical characterization of salmon by-product hydrolysate fractions (<3 kDa, >3 kDa) prepared from three different bioprocessing approaches; lactic acid (LA) fermentation; formic acid (FA) treatment; Flavourzyme (FL) hydrolysis (A) Free amino nitrogen content expressed as serine-NH_2 meqv, (B) Surface hydrophobicity (S_o).

5.3.3. In vitro antioxidant properties

5.3.3.1. Ferric reducing capacity

Ultrafiltered hydrolysate fractions (both H-MW and L-MW) were generally observed to have higher Fe(III) reducing capacity, than their corresponding crude hydrolysate fractions. H-MW hydrolysate fractions demonstrated either significantly higher or comparable Fe(III) reducing capacities, but never significantly lower than their L-MW counterparts (**Figure 5.2. A**). The L-MW fraction reducing capacity for total FL-hydrolysates were lower than unfiltered crude hydrolysate, indicating that the component responsible for the reducing activity is of higher molecular weight. Previous results showed a decrease in reducing capacity with increased processing time, beyond an initial increase for Flavourzyme treated total by-product hydrolysates (Section 4.3.7.1) also. However, L-MW fractions of T5 and V1 both showed higher Fe(III) reducing capacity. Moreover, the H-MW fraction of T2 (with higher FAN and processing duration) had significantly higher reducing capacity than T1 H-MW fraction (both prepared with Flavourzyme). Ferric reducing antioxidant potential has been generally reported in the literature,

to be higher for smaller sized peptides (protein hydrolysates with higher DH)[150] although with exceptions.[39] For instance, a study reported that <3 kDa hydrolysate fraction from Pinto bean protein hydrolysates prepared using Protamex had higher ferric reducing capacity than H-MW fractions.[165] It is evident that variations in the type of enzymatic processing can influence reducing capacity, along with molecular weight, but smaller peptide size does not always result in the highest reducing capacity.

5.3.3.2. Metal binding capacity

Fe(II) binding capacity of the FL-hydrolysates have been demonstrated to be significantly higher that the autolytic hydrolysates in the previous chapters (3.3.5.1. and 4.3.8.2.). The total by–product FL-hydrolysate increased in Fe(II) binding capacity (from 66.58±7.93% to 86.50±1.50% (L-MW fraction) following ultrafiltration. In contrast to the general trend of smaller peptide size enhancing Fe(II) chelation (correlation between FAN content and Fe(II) chelation capacity, $\rho = 0.480$, $p = 0.043$, $n = 18$),[7] the H-MW fraction with lower FAN content, exhibited higher Fe(II) binding capacity (78.36±1.03%, comparable to crude visceral FL-hydrolysate (79.71±0.94%)) than the L-MW fraction (63.98±1.92%). Additionally, L-MW T5 fractions (FA-hydrolysate) also showed a low Fe(II) binding capacity, while all other hydrolysate fractions had nil or negligible capacity to bind Fe(II).

5.3.3.3. Glutathione protection

Sacrificial antioxidant properties of the hydrolysate fractions were assessed using the glutathione protection assay.[115] A mixture of the hydrolysate fractions and GSH subjected to Fe(II)/H_2O_2 at 37° C, results in the generation of hydroxyl free radicals (Fenton's reaction) oxidizing the GSH, which reduces the free SH content in the mixture. Antioxidant peptides were expected to undergo preferential oxidation due to hydroxyl free radicals, protecting the GSH

from being oxidized to glutathione disulfide (GSSG). Higher degree of hydrolysis/ high FAN content/ smaller peptides[166,167] and lower degree of hydrolysis/ low FAN content/ larger peptides[168] has been demonstrated to be effective in hydroxyl radical scavenging in the literature.

The previous chapters of the present study indicated in general, the degree of hydrolysis to correlate negatively with the ability of peptides to quench hydroxyl free radicals and enable GSH protection. In accordance with this observation, FAN content of the fractionated hydrolysates correlated negatively with the ability to protect GSH from Fenton's oxidation (ρ = -0.800, p = 2×10^5, n = 18). In contrast to the above correlation, L-MW fractions were observed to have higher/equal GSH protection capacity (%) for most hydrolysates. The autolytic hydrolysate fractions had higher GSH protection capacity, whereas GSH was almost completely oxidized in the case of FL-hydrolysates treatment. In both LA-derived visceral (VLA) and total by-product hydrolysate (TLA) fractions, it was observed that with an increase in processing duration from 6 hours (T3, V2) to 4 days (T4, V3), the GSH protection capacity increased for the H-MW fraction bringing it closer to the GSH protection values of L-MW fractions. Variations in the GSH protection capacity of both L-MW and H-MW fractions, with the change in processing duration, indicates the importance of molecular weight distribution within each fraction. While it has been previously suggested that the Fe(II) chelation capacity could possibly enhance the ability of peptides to protect GSH from hydroxyl free radicals,[115] the Fe(II) chelation capacity of the hydrolysate fractions negatively correlated with GSH protection (ρ = -0.631, p = 0.005, n = 18).

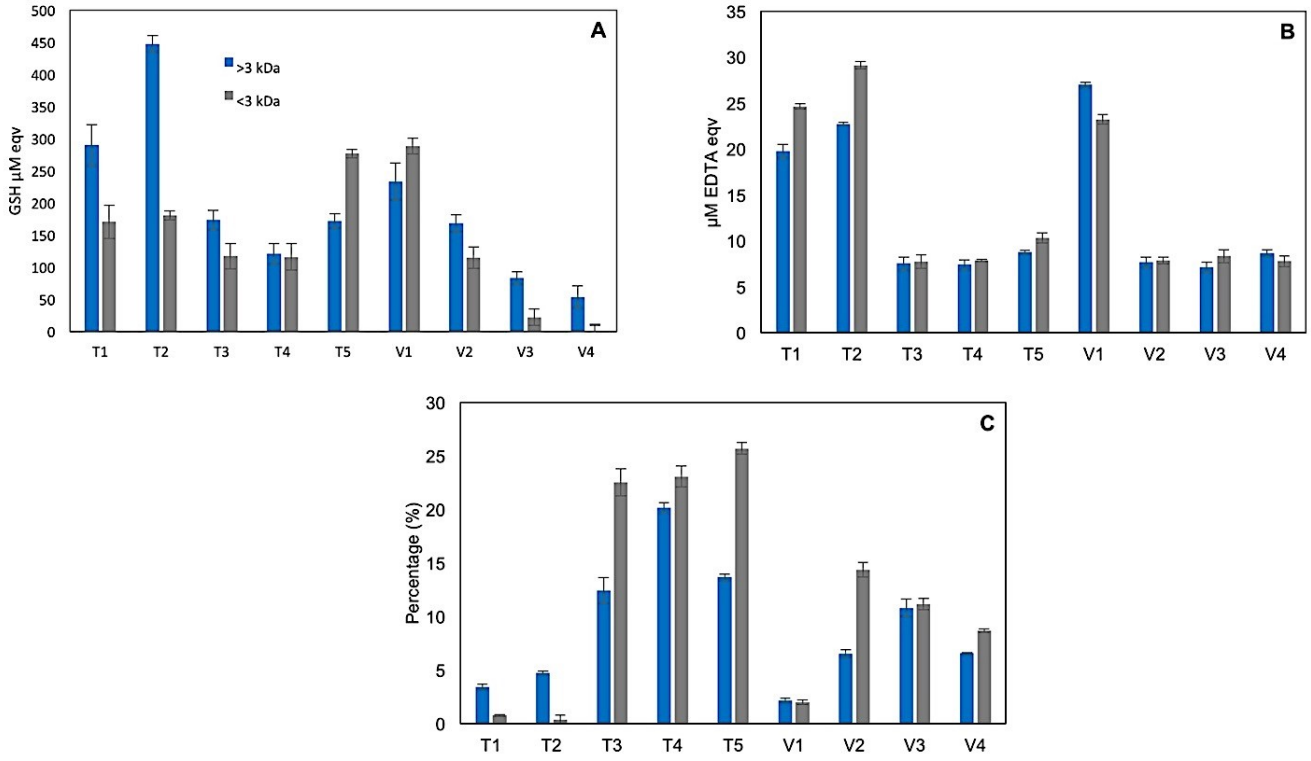

Figure 5. 2. Characterization of antioxidant activities of the salmon by-product hydrolysates in aqueous environment. (A) Ferric reducing capacity expressed as µM glutathione (GSH) equivalent, (B) Fe(II) chelation activity expressed as µM EDTA equivalent, and (C) Sacrificial antioxidant capacity of the hydrolysates assessed and expressed as percentage protection of GSH subjected to Fenton's reaction

5.3.3.4. Antioxidant properties within bovine plasma matrix: protection from H_2O_2 oxidation

Antioxidant activities within the plasma matrix provides a perspective on the antioxidant efficacy of these peptides in a complex environment with physiological significance. Protein and peptide binding in the plasma can significantly influence the distribution, pharmacokinetic behavior, availability and overall activity in living systems. Plasma components such as low-density lipoprotein, sex hormone binding globulin, α-1-acid-glycoprotein (AGP), α-2-macroglobulin, β2-microglobulin, high-density lipoprotein, haptoglobulin, fibrinogen, complement C4, haptoglobulin, α-1-antitrypsin, fibronectin, transferrin, immunoglobulin G, hemopexin, and human serum albumin have all been demonstrated to be responsible for the majority of the sequestering activity in plasma towards peptide-based drugs.[169,170] Human serum

albumin (HSA) is the most abundant and most important plasma protein followed by the critical serum protein α1-acid glycoprotein (AGP).[171]

5.3.3.4.1. Fe(III) reducing capacity in H_2O_2-oxidized plasma

H_2O_2 oxidation did not significantly influence the reducing capacity of the bovine plasma. For most samples, reducing capacity in the plasma matrix did not significantly vary between the H-MW and L-MW fractions. V2 is the only sample with statistically significant differences between the reducing capacity of L-MW and H-MW fractions, where the former was capable of enhancing the Fe(III) reduction in bovine plasma. In both visceral and total by-product LA-hydrolysates, ferric reducing capacity of treated bovine plasma increased significantly with increase in processing duration (T3 (6 hours) → T4 (4 days); V2 (6 hours) → V3 (4 days)). In a recent study using a similar plasma matrix to determine the antioxidant capacity, it was seen that peptic hydrolysates with lower DH result in higher dose-dependent increases in plasma Fe(III) reducing capacity, while pancreatic hydrolysates (higher DH) remained unable to exert any significant difference.[172] LA-hydrolysate fractions (L-MW and H-MW, derived from total by-product and viscera) were found to be most efficient at improving the reducing capacity of bovine plasma even though their aqueous suspensions had negligible Fe(III) reducing capacity.

5.3.3.4.2. Plasma sulfhydryl protection

Although the Fe(III) reducing capacity has been largely attributed to the SH content,[39] no correlation was observed between plasma protected SH levels (%) and plasma reducing capacity ($\rho = 0.091$, $p = 0.711$, $n = 18$). The H-MW hydrolysate fractions exhibited a higher capacity to protect plasma SH in general (**Figure 5.3. B**). FL-hydrolysate H-MW fractions were most efficient at maintaining plasma SH levels, a possible mechanism of their action could be *via* the

higher reducing capacity (from higher SH content), reducing the oxidized disulfide linkages in the plasma proteins. Although not significant, *in vitro* Fe(III) reducing capacity and GSH protection ability correlated positively with protected SH levels of bovine plasma (Fe(III) reduction, $\rho = 0.317$, $p = 0.195$, $n = 18$; GSH protection, $\rho = 0.291$, $p = 0.231$, $n = 18$).

5.3.3.4.3. Change in GSH content of hydrolysate treated oxidized plasma

In general, it was observed that the visceral hydrolysate fractions were less efficient in improving the GSH content in the plasma (**Figure 5.3. C**). Additionally, FA-hydrolysate fractions (both total by-product and viscera derived) performed poorly in increasing plasma GSH contents. It is believed that residual FA contents may have played a role in additional oxidation observed in V4. On the other hand, the significantly higher plasma-GSH protection capability of T2 can be attributed to the high reducing capacity (**Figure 5.2. A**). It is noteworthy to mention that T2 was able to protect and even enhance the plasma-GSH content, more than in the unoxidized plasma. Change in treated plasma-GSH content positively correlated (not significant) with the aqueous Fe(III) reducing capacity ($\rho = 0.430$, $p = 0.072$, $n = 18$).

5.3.3.4.4. Protection against plasma lipid peroxidation

H-MW hydrolysate fractions significantly protected the plasma from the generation of lipid peroxides compared to the L-MW counterparts (**Figure 5.3. D**). In another study, it was reported that casein calcium peptides of major mass distribution of around 3 kDa effectively limited lipid peroxidation in ground beef homogenates, while also exhibiting high free-radical ($OH^{\bullet}$, $O2^{\bullet}$, DPPH) scavenging activities *in vitro*.[173] H-MW fractions of FL-hydrolysates were especially effective in lipid peroxide inhibition. This is in agreement with a previous report of soy protein hydrolysates prepared through Flavourzyme treatment, efficiently inhibited lipid peroxidation in a liposome-oxidizing system as compared to hydrolysates prepared with pepsin,

papain, Protamex and Alcalase.[174] In yet another report, protection against lipid peroxidation was assessed in salmon fillets glazed with Pollock skin hydrolysates, where hydrolysate with lower FAN content (presumably larger peptides) provided a higher degree of protection.[98] In the present study, although the L-MW fractions of T1 and T2 were able to chelate Fe(II) more, they were significantly less effective in preventing plasma lipid peroxidation. This observation was in accordance with the other reports, where incorporation of protein hydrolysates with higher metal chelation activity into ground beef did not significantly enhance lipid peroxide inhibition.[175,176]

Therefore, the role of *in vitro* Fe(II) chelation capacity for enabling antioxidant mechanisms for peptides in a complex living environment needs further validation. Moreover, it was observed that other parameters of plasma antioxidant activity correlated positively (except for plasma SH content, not statistically significant) with protection against lipid peroxidation (plasma Fe(II) reduction, $\rho = 0.321$, $p = 0.189$, $n = 18$; plasma SH content, $\rho = 0.552$, $p = 0.017$, $n = 18$; plasma GSH content, $\rho = 0.197$, $p = 0.426$, $n = 18$).

While it was evident that the L-MW fractions were more effective in the aqueous *in vitro* antioxidant assays (**Figure 5.2.**) based on the comparison between plasma based assays (**Figure 5.3.**) it can be argued that the <3 kDa peptides are less effective in complex environments similar to bovine plasma in effectively exerting antioxidant activities.

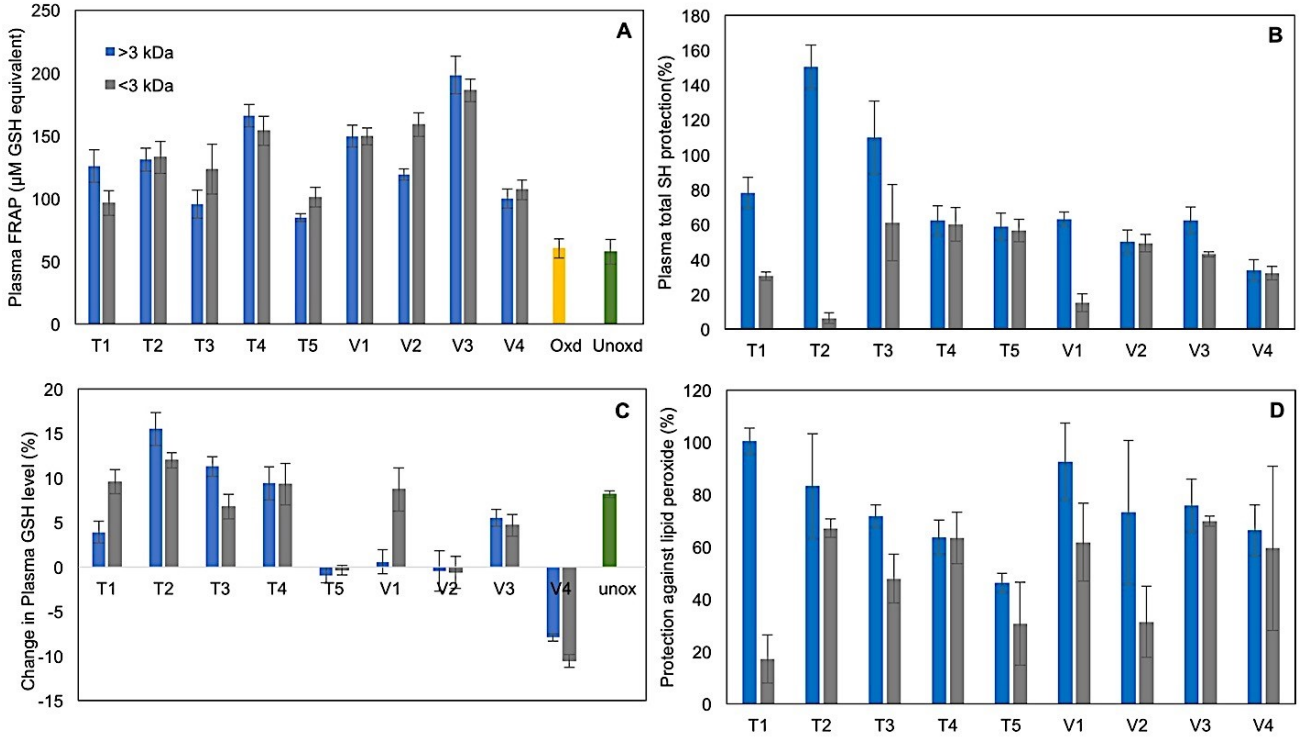

Figure 5. 3. Characterization of antioxidant activities of the salmon by-product hydrolysate fractions (<3 kDa, >3 kDa) in bovine plasma matrix (subjected to H_2O_2/Fe(II) (50 µM) oxidation (2 hours)). (A) Ferric reducing capacity expressed as µM glutathione (GSH) equivalent, (B) Protected plasma sulfhydryl content (%), and (C) Change plasma GSH content (%) from oxidized plasma, (D) Protection against plasma lipid peroxidation (%).

5.3.3.5. Intracellular antioxidant capabilities

5.3.3.5.1. Cytoprotection

FL-hydrolysate (T1, T2 and V1) fractions in general, were more efficient in protecting the HT29 cells against oxidative stress (**Figure 5.4.**). In contrast, the visceral FA-hydrolysate fractions (V4) were observed to be pro-oxidant in the intra-cellular systems which is in line with the results in the plasma matrix. T1, T2 (L-MW), V1 and V3 (L-MW) were observed to have a concentration dependent increase in cytoprotection capacity, except for the highest concentration (0.5 mg/mL). Both H-MW and L-MW fractions at certain concentrations were able to protect the cells and even stimulate higher growth. Similar studies have been able to show concentration

dependent cytoprotection capacity of salmon protein hydrolysates.[177] For instance, treatment with salmon FA-protein hydrolysates have been reported to result in the reduction of catalase expression and caspase-3 expression and activation in cultured, oxidative-stressed kidney cells, indicating that the these peptides can quench the reactive capacity of H_2O_2.[178] On the other hand, unstressed kidney and liver cells treated with salmon FA-hydrolysate demonstrated increased expression of catalase and caspase-3.[159]

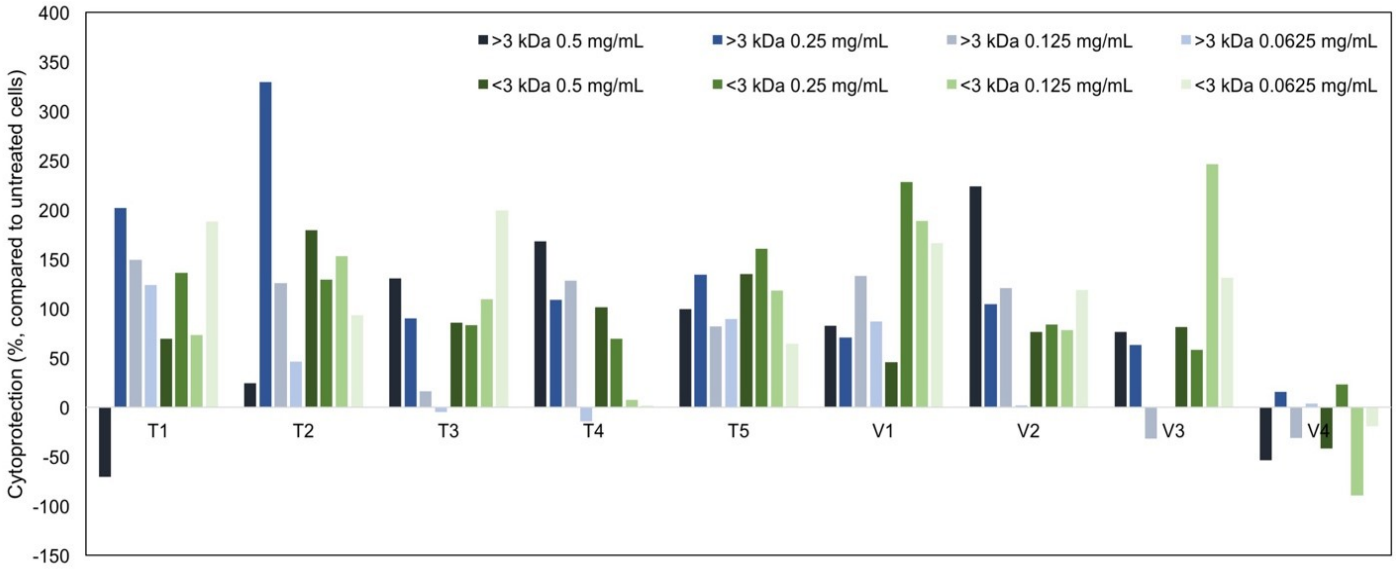

Figure 5. 4. Cytoprotection capacity of pretreated salmon by-product protein hydrolysate fractions (<3 kDa, >3 kDa) on HT29 cells subjected to H_2O_2 (200 µM) stress.

The cytoprotection capacity at the higher concertation of treatment (0.5 mg/mL), correlated positively with the aqueous GSH protection capacity ($\rho = 0.657$, $p = 0.002$, $n = 18$) while at lower concentrations, the correlation was negative (0.25 mg/mL, $\rho = -0.273$, $p = 0.266$, $n = 18$; 0.125 mg/mL, $\rho = -0.337$, $p = 0.167$, $n = 18$; 0.0625 mg/mL, $\rho = -0.430$, $p = 0.072$, $n = 18$). Therefore, at higher concentrations the scavenging capacity and sacrificial antioxidant activity seem to play the dominant role. In contrast, aqueous Fe(III) reducing capacity of the treated samples correlated positively with cytoprotection at lower concentrations (0.25 mg/mL, ρ

= 0.659, p = 0.002, n = 18; 0.125 mg/mL, ρ = 0.447, p = 0.061, n = 18; 0.0625 mg/mL, ρ = 0.598, p = 0.008, n = 18). Most of the plasma based antioxidant parameters correlated positively (not statistically significant) with the cytoprotection capacity of the hydrolysate fractions, particularly at lower concentrations.

5.3.3.5.2. Intracellular GSH levels

Lower levels of intra-cellular GSH have been suggested to not be the major cause of apoptosis, while protein thiols were shown to play a relatively more significant role in this regard.[179] However, GSH does have a critical role in regulating the redox status of these protein thiols within the cells. It was observed that for unstressed HT29 cells treated with hydrolysates, the GSH levels were generally lower than hydrolysate treated stressed cells (**Figure 5.5.**). Additionally, visceral hydrolysate fraction treated unstressed cells were observed to have increased levels of GSH. Phe-Cys dipeptide has been demonstrated to increase the GSH levels in cultured unstressed human hepatocytes.[163] Another similar report on rat hepatocytes treated with H_2O_2 found Chum salmon hydrolysates to increase (not statistically significant) GSH levels.[177] In contrast, casein phosphopeptides (CPPs) preincubated Caco-2 cultures subjected to H_2O_2 stress have demonstrated to decrease intracellular GSH.[180] It is noteworthy to mention that in the above study, Caco-2 cells subjected to 5 mM treatment did not significantly change the GSH content from unstressed cells.[181] Another study using H_2O_2 (5 mM) stressed Caco-2 cells treated with CPPs, however, found oxidative stress to reduce GSH levels, while CPP treatment increased GSH concentrations.[182] These studies suggest free radical scavenging and metal chelation as the primary mechanisms underlying the intracellular antioxidant activities of these peptides.[181,180,182]

In the present study, Fe(II) chelation capacity correlated positively with the intracellular GSH levels in H_2O_2 stressed (50 μM) HT29 cells (0.5 mg/mL, ρ = 0.459, p = 0.054, n = 18; 0.25

mg/mL, ρ = 0.325, p = 0.183, n = 18). While on the other hand, aqueous GSH protection capacity negatively correlated with the intracellular GSH levels of both stressed and unstressed cells (statistically not significant) treated with the protein hydrolysates (0.5 mg/mL (s), ρ = -0.752, p = 710^{-5}, n = 18; 0.25 mg/mL (s), ρ = -0.695, p = 0.001, n = 18; .5 mg/mL (un), ρ = -0.282, p = 0.252, n = 18; 0.25 mg/mL (un), ρ = -0.091, p = 0.711, n = 18). It is however, unclear whether the increase/decrease in GSH levels observed between stressed and unstressed cells were a result of synthesis, protection, regeneration or depletion of intracellular GSH.

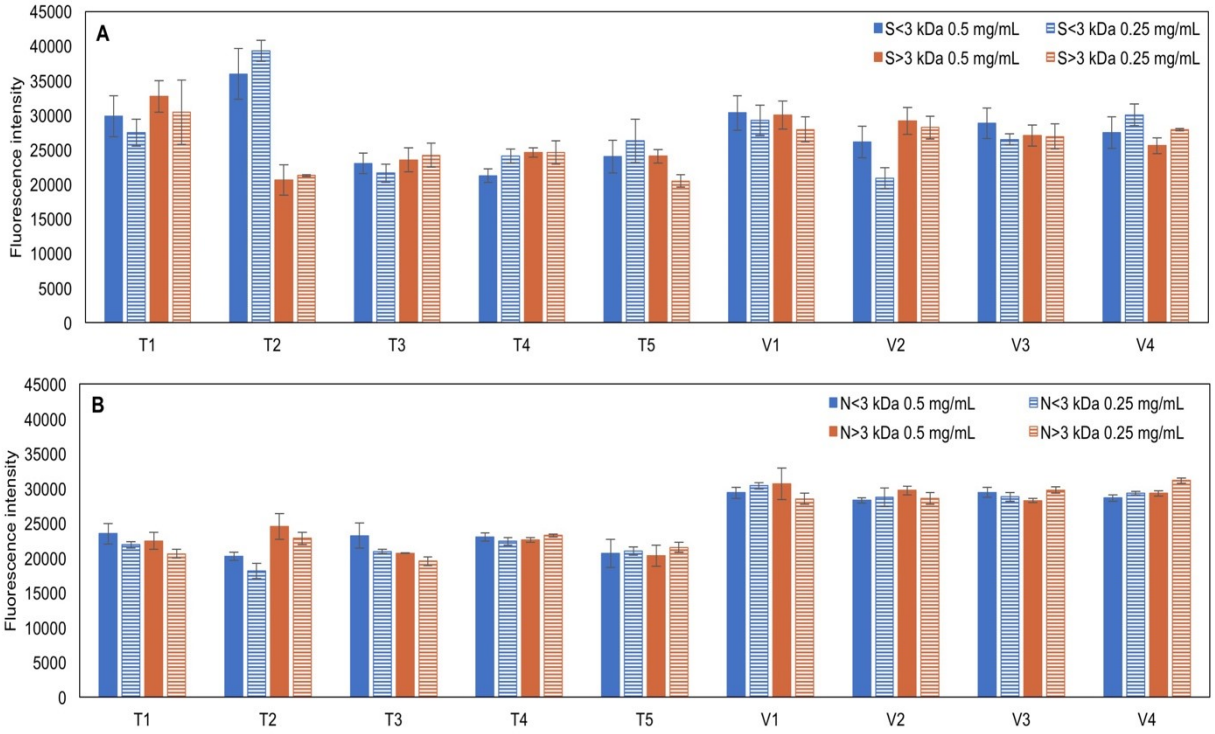

Figure 5. 5. Intracellular GSH levels as indicated by fluorescence intensity following pretreatment with salmon by-product protein hydrolysate fractions (<3 kDa, >3 kDa) pretreated on (A) HT29 cells subjected to H_2O_2 (50 µM) stress and (B) unstressed HT29 cells.

5.3.3.5.3. Intracellular lipid peroxidation

As expected, unstressed cells had lower levels of lipid peroxide levels (**Figure 5.6.**). Although not statistically significant, the lipid peroxide levels in sample treated cells (stressed

and unstressed) correlated consistently with aqueous GSH protection capacity of the hydrolysate fractions (0.5 mg/mL (s), ρ = -0.164, p = 0.508, n = 18; 0.25 mg/mL (s), ρ = -0.042, p = 0.863, n = 18; 0.5 mg/mL (un), ρ = -0.554, p = 0.016, n = 18; 0.25 mg/mL (un), ρ = -0.152, p = 0.541, n = 18). T3 and T4 (LA-hydrolysates) L-MW fraction treated cells showed the highest dose-dependent protection against intracellular lipid peroxidation (**Figure 5.6.**). Similar results were reported in a study using chum salmon hydrolysates prepared with Alcalase and papain, which reduced the intracellular lipid peroxidation in a dose-dependent manner and hydrolysates prepared with a higher DH provided better protection.[177] V4 treatment resulted in an increase in lipid peroxidation, consistent with lower antioxidant capacity (pro-oxidant in certain cases) observed in aqueous, plasma and cellular assays.

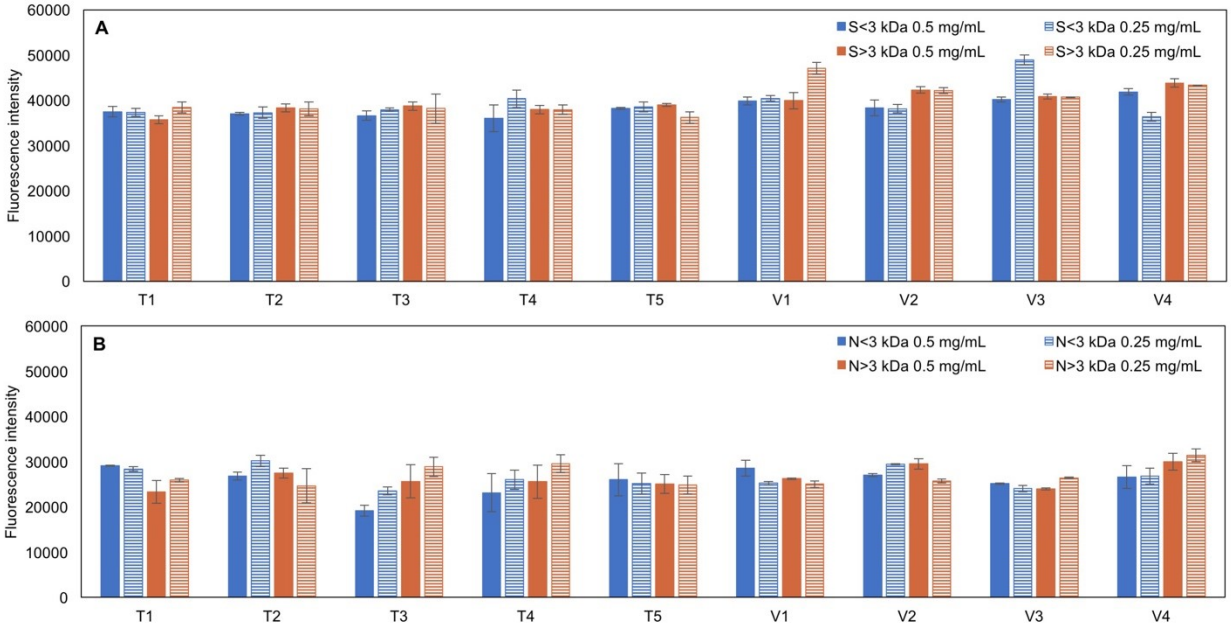

Figure 5. 5. Intracellular lipid peroxide levels as indicated by fluorescence intensity following pretreatment with salmon by-product protein hydrolysate fractions (<3 kDa, >3 kDa) pretreated on (A) HT29 cells subjected to H_2O_2 (50 µM) stress and (B) unstressed HT29 cells.

5.4. Conclusion

The ultrafiltered visceral hydrolysate fractions had similar properties with corresponding crude unfractionated visceral hydrolysates. In contrast, ultrafiltration significantly influenced the properties of total by-product hydrolysate. While the <3 kDa fractions were more promising based on the aqueous bioassays, in the plasma matrix, the >3 kDa hydrolysate fractions demonstrated higher antioxidant capacity in general. Moreover, based on the intracellular antioxidant capacities, neither molecular weight fractions can be associated with better antioxidant effects. Processing parameters and enzymatic cleavage specificity clearly played a major role in underlying antioxidant mechanisms of peptides. While the Flavourzyme derived hydrolysates demonstrated higher metal chelation and reducing capacity, autolytic hydrolysates proved to be effective in radical scavenging and preventing intracellular lipid peroxidation. Hydrolysate fractions were successful in general, in suppressing the oxidative events in HT29 cells as well as the plasma. The present study also demonstrated the antioxidant abilities of hydrolysates derived from fish processing waste streams in different physiologically relevant models highlighting their potential as functional food ingredients in health and storage applications.

CHAPTER 6

CONCLUSION

Atlantic salmon (*Salmo salar*) is an integral part of the seafood and aquaculture industry in Canada and significantly contributes to the Maritime provinces. Statistics Canada report estimates Canadian exports of Atlantic salmon in 2014 to be around 300,000 metric tonnes. Salmon processing waste has been shown to be a very good source of proteins, however solid wastes from these industries are disposed or dumped in landfills or the ocean. This is an expensive and wasteful approach for the processing industries as well as the environment. Furthermore, the processing products are underutilized as fish meal to feed animals.

The present study was aimed at extracting proteins present within the salmon by-products using different approaches. The influence of different bioprocessing approaches were assessed on salmon viscera and total by-products mix. The influence of two autolytic approaches involving formic acid treatment and lactic acid fermentation were compared to enzymatic proteolysis-processing using the commercial protease preparation Flavourzyme. While autolytic processing approaches were characterized to rely upon organic acids to lower pH and activate proteases, Flavourzyme was added exogenously. The processing approaches utilized in this study simplifies and integrates the steps involved in by-product protein hydrolysate preparation from fish by-product, to ensure applicability during scale-up and utilized inherent moisture in the ground salmon tissues. The lactic acid bacterial growth and metabolic features were also characterized within the salmon by-product and salmon viscera substrates.

As expected, Flavourzyme demonstrated high proteolytic activity even though the activity steadily decreased with time. For the autolytic processing, enzymatic activity varied significantly between salmon viscera (higher protease content) and total by-product mix. While

the fermentation resulted in increase of protease activity over time, the opposite was observed for formic acid treatment. Additionally, the degree of protein hydrolysis during the fermentation was found to be on par with Flavourzyme processing as well. However, both the autolytic approaches were carried out for a longer processing duration of 8 days, with no observable fouling, while the Flavourzyme application was limited to 12 hours. Each approach was unique in the recovery or yield and each imparted significantly different functional properties to the protein hydrolysate. Formic acid treatment was generally more efficient in total soluble protein recovery followed by lactic acid fermentation.

Commercial proteases such as Flavourzyme have benefits in terms of a uniform cleavage pattern that maintains uniform functional characteristics, thereby contributing to enhanced antioxidant activity namely higher Fe(II) chelation and ferric reducing capacity. The autolytic hydrolysates demonstrated higher sacrificial antioxidant activity. The results from this study also showed that lower molecular weight cannot always be associated with higher antioxidant capacity in all the matrices, which has been the general trend in the literature. Although, antioxidant properties of lower molecular weight ultrafiltered salmon by-product protein hydrolysate, had promising antioxidant activity in the aqueous bioassays. However, higher molecular weight hydrolysate fractions demonstrated higher protective effects within the relatively complex bovine plasma-matrix. Moreover, neither low or high molecular weight fractions were found to be better than the other in the cellular (HT29) model subjected to hydroxyl radical-mediated oxidative systems while both exhibited significant protective intracellular antioxidant capacities.

Fractionation on the basis of molecular weight had a greater influence on total salmon by-products than on their visceral counterparts, since the activities of fractionated and unseparated

hydrolysate was comparable for the latter, but significantly different for the former. Thus, the current study has shown the antioxidant capabilities of salmon protein hydrolysate in multiple physiologically relevant models, providing a comprehensive evidence on potential applications as ingredients in functional health formulations and food storage applications.

Outcomes from the present study help in improving our understanding of the influence different proteolytic approaches have on recovery and antioxidant functionality of fish-based hydrolysates in general. Salmon by-product protein hydrolysate generated *via* enzymatic, chemical and microbiological processes from the perspective of commercial production can thus, open further possibilities in effectively utilizing these waste streams for functional food/feed development or in improving food storage. Further research will be required to elucidate nutritional, textural, and sensory changes associated with salmon by-product preparation and incorporation into food matrices.

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