CHARACTERIZATION OF PEPTIDE FRACTIONS FROM ATLANTIC SALMON (*Salmo salar*) BY-PRODUCT FERMENTATE: EVALUATION OF COMPOSITION, STABILITY, SELECTIVITY AND RECOVERY DURING FRACTIONATION USING ELECTRO-ULTRAFILTRATION

by

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ABSTRACT

Valorization of fish by-products has inherent economic and environmental advantages. Not only are these viable sources of biomolecules with nutritional value (edible proteins and fish oil), fish by-products are also reservoirs of functional molecules with applications in food ingredient manufacturing and human health. Downstream processing is the costliest step in product recovery, limiting the commercial development of functional foods and nutraceuticals from alternative sources. During upstream processing of salmon by-products, endogenous enzymes in the tissues facilitated the hydrolytic release of oil, peptides and other mineral components in the fish tissue. Centrifugal separation of the by-product fermentate resulted in four chemically distinct fractions: oil, emulsion, aqueous and residue. Fish oil and aqueous protein extracts have been well characterized in the literature. In the present study, the emulsion phase (lipopeptide complex) was characterized along with the aqueous phase consisting of salmon protein hydrolysate (SPH). The emulsifying ability of the peptides in the emulsion fraction is attributed to the higher abundance of hydrophobic amino acids. Crude SPH needs further separation for higher value applications such as functional food ingredient. In this light, shelf life and storage stability of SPH was evaluated and components were found to be largely unaltered over the course of 12 weeks at 4 °C. Downstream processing strategies enable efficient enrichment of molecules of interest from SPH. Membrane filtration has the ability to deliver high throughput and scalable processing, but also exhibits limitations such as fouling and low selectivity. To overcome this, a filtration approach combining pressure and applied voltage called electro-ultrafiltration (EUF) was used. EUF resulted in improved recovery and selectivity during processing. Higher abundance of charged amino acid residues in electro-ultrafiltered fractions allowed these fractions to exhibit distinct functional properties, such as high metal chelation capacity. These findings are relevant to understanding the composition and behavior of peptides derived from fermentative processing of fish by-products, throughout sample processing, storage and fractionation. This research contributes towards our understanding of the peptide physicochemical properties that determine functional activity. Research relevance also extends to novel product development from underutilized resources such as fish by-products, which is especially relevant to Atlantic Canada.

LIST OF ABBREVIATIONS AND SYMBOLS USED

Terms/abbreviations	Definitions
<3A	<3 kDa anionic fraction
<3C	<3 kDa cationic fraction
<3N	<3 kDa neutral fraction
ABTS	2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid
ACE	Angiotensin-converting enzyme
Ala	Alanine
ANOVA	Analysis of variance
Arg	Arginine
Asp	Aspartate
Asx	Aspartate + Asparagine
BSA	Bovine serum albumin
C_B	Concentration of solute in bulk feed
C_P	Concentration of solute in permeate
C_W	Concentration of solute in membrane wall/surface
CFU	Colony forming unit
CID	Collision induced dissociation
Da	Dalton
DH	Degree of hydrolysis
DPP4	dipeptidyl peptidase IV
DPPH	2,2-diphenyl-1-picrylhydrazyl

DPPP	1,3-Bis(diphenylphosphino)propane
EDR	electrodialysis reversal
EDUF	electrodialysis with ultrafiltration membranes
E/S ratio	Enzyme to substrate ratio
EDTA	Ethylenediaminetetraacetic acid
EUF	Electro-ultrafiltration
F	Conversion factor of 6.25 used in protein content calculation
FAN	Free amino nitrogen content
FRAP	Ferric reducing antioxidant potential
GC-FID	Gas chromatograph-Flame ionization detector
GFC	Gel filtration chromatography
Glx	Glutamine + Glutamate
Gly	Glycine
GMP	Good manufacturing practices
GRAVY	Grand Average of Hydropathicity Index
GRAS	Generally regarded as safe
GSH	Glutathione
HACCP	Hazard analysis and critical control point
HPLC	High performance liquid chromatography
J_V	Filtrate flux
LAB	Lactic acid bacteria
LCD	limiting current density
LC	Liquid chromatography

LC-MS/MS	Liquid chromatography - tandem mass spectrometer
LC-UV	Liquid chromatography with a variable wavelength detector
L_P	Hydraulic permeability coefficient
LTQ	Linear Trap Quadrupole
m/z	Mass to charge ratio
μ_{ep}	electrophoretic mobility
MRPs/iMRPs	Maillard reaction products/ intermediate Maillard reaction products
MW	Molecular weight
NF	Nanofiltration
PES	polyethersulfone
Phe	Phenylalanine
pI	Isoelectric point
PITC	Phenyl isothiocyanate
PSMs	Peptide spectral matches
QSAR	Quantitative structure-activity relationship
r^2	Correlation coefficient
ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
SCX	Strong cation exchange
SPE	Solid Phase Extraction
SPH	Salmon protein hydrolysate

S_o	Observed sieving coefficient
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
Tyr	Tyrosine
UF	Ultrafiltration
UPLC	Ultra-Performance liquid chromatography
VWD	Variable wavelength detector
WAX	Weak anion exchange
η	viscosity

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CHAPTER 1

Introduction

1.1. Bioactives from marine processing co-product streams

There is a global push towards using naturally derived ingredients in the food, animal feed and petfood industries. Food components referred to as bioactive ingredients can play a significant role as health-benefiting factors to help alleviate disease conditions and stress. Atlantic Canada is host to an emerging bioeconomy based on the production of functional biomolecules or bioactive compounds which can impart beneficial effects for animal, plant, and human health. While some of these bioactive compounds such as polyphenols, seaweed extracts, omega-3 lipids, and whey protein hydrolysate are already commercialized, a growing demand exists to develop new classes of bioactives as well as to improve the efficacy of existing heterogenous products.

The development of novel bioactive ingredients and sustainably-sourced bioproducts that do not compete with current food supply chains is essential for diversifying the food systems, especially from the perspective of an increasing population that is predicted to reach 9.7 billion in 2050.[1] To this end, management of waste streams from various agricultural and food-related industries is essential to reduce the environmental burden and enhance value creation for niche markets for otherwise underutilized resources. 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. Especially relevant to Atlantic Canada are waste streams generated from fish

processing. In our region between 2000 and 2011, it was estimated that ~700,000 tonnes of fish processing waste were disposed into the ocean.[2] This stems from the highest number of waste disposal permits issued in Canada, with >400 permits at the time.[2] These by-product streams constitute approximately 60% of the input by mass used in fish processing industries.[3] In the case of wild-caught fish, by-products are being used for fish oil production, which is an important first step towards limiting waste production and increasing economic utility. However, the edible protein components of fish waste remain underutilized. In farmed and wild-caught fish, the processing by-products should be viewed as expansive reservoirs of quality edible proteins and a source for production of bioactive peptides, which can augment the economic value of these by-products beyond use as low commodity fish meal or fertilizer.[4] Therefore, there needs to be additional emphasis on developing efficient approaches to utilize these rich sources of protein for human consumption. Utilization of these by-products will have economic and environmental benefits, enhancing the sustainable use of marine products. A great diversity of traditional preparations using seafood involves enzymatic hydrolysis, bacterial/fungal fermentation or chemical (salt/acid) treatment.[5] In that light, the group at the Verschuren Centre (Sydney, NS) has previously developed a scalable microbial bioprocessing approach to generate fish protein hydrolysate from fish by-products.[6–8] This presents a stepping stone towards further separation of bioactive peptides with desired properties.

Biologically active compounds with enhanced functionalities can contribute towards the development of high value products. Several studies have demonstrated peptides generated from marine sources of proteins to function as potential antioxidative agents.[9–12] In fact, numerous food-derived (fish, meat, milk and soybean proteins) bioactive peptides have been shown to potentially positively contribute towards managing health conditions such as hypertension,[13] thrombosis,

type-2 diabetes, cancer,[14] and osteoporosis.[15] In addition to their health benefits, bioactive peptides can also have applications towards enhanced shelf-life of food materials via bacteriostasis and antioxidant activity.[15] These activities are dependent on peptide structure, molecular interactions (behavior) and physicochemical properties which are reliant on the amino acid composition.[15]

Incorporation of peptide ingredients into food or feed formulations inherently results in a lower dosage of these bioproducts. Therefore, enrichment strategies can play a significant role in ensuring the selective higher abundance of peptides of specific properties thereby enhancing activity at low dosage, which is necessary for development of bioproducts that can influence positive health outcomes in food formulations. In this regard, membrane-based separation technologies have the potential to reduce operation costs for fractionation and enrichment while incorporating multi-product processing capabilities. Ultrafiltration and nanofiltration commonly used in dairy processing are generally pressure-driven processes by which macromolecules are selectively separated based on their molecular size. However, this approach cannot be used to separate molecules of similar size ranges. Membrane fouling is another drawback commonly associated with filtration-based processing approaches (Chapter 4).

This chapter will focus on introducing the approaches used in peptide based bioproduct development in food and feed applications. Various aspects to consider for the development of effective bioprocess/bioproduct development strategies will be described, such as raw materials, scale-up, analytical characterization, storage, peptide modifications and functionality of the hydrolysates.

1.2. Considerations towards use of fish by-products: Protein isolation

Raw materials used in product formulations (by-products from fish processing) play a major role in determining quality and functionality. The use of wild caught fish by-products is

determined by parameters such as freshness of product at processing, nutritional profile and availability of cold storage. Aquaculture production has now surpassed wild catch and by-products generated from the aquaculture sector exhibit lower variability.[16,17] Fish by-products have been shown to have large variations in the composition and protein content for the same species harvested and processed at different locations. For example, Atlantic salmon (*Salmo salar*) by-product yields from Chile[3] demonstrated significant differences when compared to those caught and processed in South Australia.[18] In addition to geographic and seasonal variations, raw material storage also influences the protein quality. For example, Šližytė *et al*. (2009) evaluated the influence of storage (of raw materials) and preparatory conditions on the yield and functionality of cod backbone hydrolysates.[19] Fresh raw materials resulted in higher yields and better emulsification properties of the hydrolysate.[19] Therefore, it can be summarized that variations in substrate (raw materials) quality and processing parameters can influence bioproduct functional properties.

1.3. Fish protein concentrate production

Fish tissue contains an abundance of food grade proteins that have nutritional value (protein supplement) and can also be used for development of novel ingredients. Protein isolates or concentrates are prepared by the separation of protein from other tissue components. Production of protein concentrate conventionally precedes preparation of fish protein hydrolysate. Methods that utilize protein precipitation, following pH shifts are extensively used in the preparation of protein concentrates/isolates from fish by-products.[20,21] For instance, fish heads were used to prepare protein isolates following homogenization with high ratios of distilled water used in addition to pH shift with NaOH or HCl.[22] A consideration towards the preparation of protein concentrates is the impact of processing on functionalities (emulsifying ability, solubility etc.).

Alkaline extraction resulted in protein isolates with better solubility and gel forming ability while acidic isolates exhibited higher surface hydrophobicity.[22] A similar study conducted with tilapia frame by-products demonstrated the highest protein recovery at pH 12 with a high solvent ratio of 9:1.[23] Using this approach, however, very high amounts of lye would be needed to process a ton of fish by-products at pilot scale, which is not sustainable. Additionally, alkali agents induce disulfide linkages as prolonged exposure to higher pH results in deprotonation of sulfhydryl groups (pKa of S-H is 8.3) and oxidation forming disulfide bonds, in turn leading to protein aggregation, thereby reducing recovery.[24] Generally, sustainable processing approaches should be relying less on harsh acidic or alkali treatment for the extraction of proteins from fish by-products.

Figure 1.1 depicts a conventional workflow for the production of fish protein hydrolysate. In this approach, fish protein isolation is followed by hydrolysis to generate peptides. This multistep process can be expensive and input intensive (time, energy and chemicals). Production of protein concentrate need not always precede production of protein hydrolysate. The bioproducts group at the Verschuren Centre previously demonstrated that soluble protein hydrolysate can be prepared directly from a by-product slurry using chemical, enzymatic and microbial approaches.[8] The low input bioprocessing approach does not use additional moisture or enzymes to carry out the proteolysis of fish tissue. The approach relies on inherent hydrolytic enzymes activated as a result of low pH from metabolic activity of lactobacilli within the fish tissues to hydrolyze and release moisture, peptides, oil etc. into the aqueous matrix. Whereas several studies that generate protein hydrolysate/concentrate[12,21,25–27] from marine by-products report the addition of water.[28–34] This addition of water during processing and its removal by freeze/spray drying is an operational expense that may be a barrier for the commercialization of the protein hydrolysate.[35] Moreover, lengthy processing times for protein concentrate production is associated with the formation of

protein degradation products and loss in nutritional quality,[36] which is not ideal. Therefore, bioprocessing approaches that are relatively quick, low input (water, enzymes, energy, and chemicals) and using a biorefinery approach (reduce wastage) are the most likely to be commercially viable for production of fish protein isolates/concentrates.

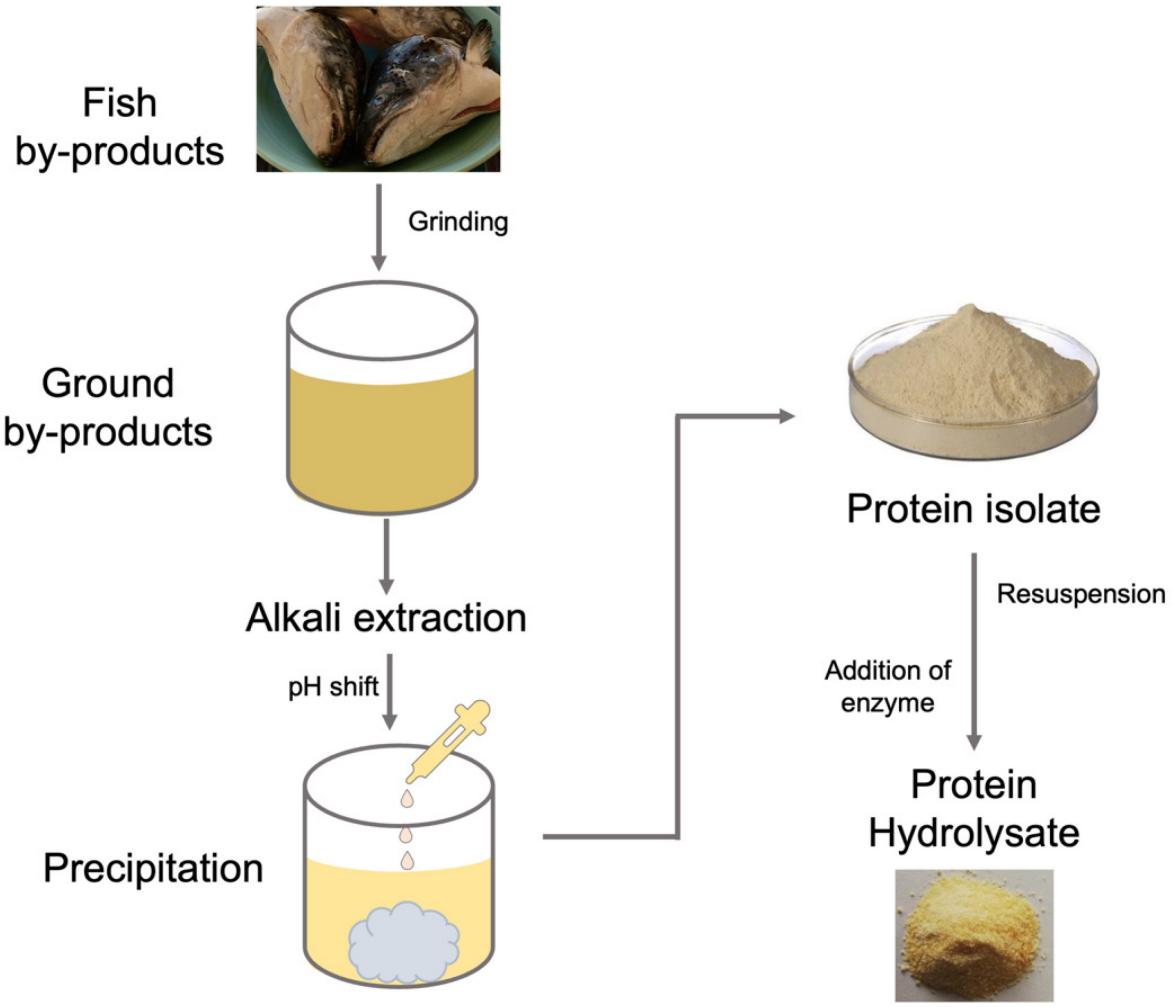

Figure 1.1: *Flowchart describing the steps involved in the production of fish protein hydrolysate from fish by-products via enzymatic hydrolysis*

1.4. Production of marine by-product protein hydrolysate

A protein hydrolysate is a mixture of peptides of varying molecular weight and composition and is commonly generated from food proteins via hydrolysis of peptide bonds at different locations. A plethora of enzymatic, biological and chemical methods have been employed for the production of protein hydolysates and bioactive peptides.[37–40] Hydrolytic specificity plays a major role in determining the makeup of peptides along with the amino acid composition and conformation of parent protein. Protein recovery has been shown to be impacted positively with higher degree of hydrolysis (DH), which is a measure of the extent of proteolysis in a given sample.[41] Higher DH improves solvent accessibility and breakdown of intact tissue components. The process of hydrolysis influences physicochemical properties such as molecular size, hydrophobicity and polar groups of the protein hydrolysates.[35] Protein hydrolysates that are generated with a higher DH have been shown to demonstrate higher solubility,[42] recovery and antioxidant activity.[12] In contrast, a few studies have also reported the antioxidant properties of fish by-product derived native peptides which have not been hydrolyzed.[43,44] A high DH is also associated with bitter taste as a result of exposed aromatic amino acid residues and reduced surface hydrophobicity (interfacial) properties (e.g., emulsifying activity) in salmon (*Salmo salar*) protein hydrolysates.[45] Longer processing times tend to favor higher DH and in turn to better yields.[19] 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 (an assay to measure radical scavenging capacity) or lipid peroxide inhibition in a liposome model.[19] Better interfacial properties of hydrolysates have been previously observed in higher molecular weight peptides, i.e. when DH is low.[27] Therefore, it can be summarized that recovery increases with DH, while the impact of DH on functionality can vary.

The physicochemical properties and potential physiological bioactivities of bioactive peptides 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, utilizing generally regarded as safe (GRAS) materials and proper food handling is crucial in product development. 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,[46] cross-linkages, chemical-modifications,[47] or isomerization (racemization).[48] The following sections describe effective approaches, namely enzymatic and microbial approaches, to generate protein hydrolysates for food applications.

1.4.1. Enzymatic preparation of hydrolysates

Commercial enzymes are typically a purified form of a biological extract. Crude biological extracts can be derived from fish or other by-products and are hence cheaper alternatives to purified enzymes in process workflows. Digestive enzymes and enzymatic extracts consist mostly of analogs of pepsin, trypsin and chymotrypsin. These proteases have narrow cleavage-specificity as compared to commercial broad spectrum microbial protease preparations.[49,50] Peptides generated with this approach are most likely to survive conditions in the gastrointestinal tract when consumed. For instance, multifunctional sea cucumber hydrolysates prepared with pepsin and tryptic hydrolysis was demonstrated to be resistant to simulated gastrointestinal digestion.[50] Because of the narrow cleavage specificity, processes with digestive enzymes were expected to have relatively low DH. On the contrary, Atlantic salmon pyloric caeca extract was demonstrated to have comparable DH as commercial alkaline proteases (Alcalase, Flavourzyme, Corolase).[31]

Another report comparing several enzymes (Alcalase, Flavourzyme, Neutrase, pepsin, Protamex, and trypsin) found protein recovery to have not varied (~10%).[51] Additionally, the concentration of viscera extract (influencing the E:S (enzyme:substrate) ratio), as well as the duration of hydrolysis and DH were each demonstrated to be closely correlated with the protein recovery.[52] Crude pepsin prepared by autolysis of the mucous membranes of sheep stomach was effectively used for pilot scale processing of fish solid waste resulting in a 50% recovery of protein hydrolysate.[53] Therefore, crude digestive enzyme preparations are suitable for the development of cheaper, sustainable and effective proteolytic processing. Typically, the recovery of protein hydrolysate can summarily depend on the substrate quality (particle size distribution of ground fish tissues, type of tissue and its abundance (e.g., skin is more resistant to proteolytic degradation), E:S ratio,[54] temperature,[55] DH,[56] processing duration[56] and solvent: substrate ratio. From the bench scale to commercial production, protein recovery is expected to vary significantly depending on these parameters. Some parameters such as amount of enzymes added (E:S ratio) can play a more significant role. For example, an increase in the concentration of protease added to the processing resulted in a 4-fold increase in soluble nitrogen in the extract.[57] These factors play complex roles during protein hydrolysate preparation. For instance, exposure to higher temperature (60 °C) during processing facilitates denaturation of structural proteins (collagen and sarcoplasmic proteins (myoglobin)) in fish tissues, thereby enabling increased hydrolysis.[58–60] However, higher temperature may also impact the activity of endogenous proteases and result in chemical modifications.[49,61] Exposure to higher temperature can also induce chemical interactions such as the formation of Maillard reaction products (MRPs; high temperature along with higher pH) and lipid peroxidation.[62] However, cost limitations associated with inputs such as amount of enzymatic

preparations or heat associated with maintaining a higher temperature should also be taken into account for commercialization of enzymatically bioprocessed fish by-product protein hydrolysate.

1.4.2. Microbial/fermentative preparation of hydrolysates

Traditional fish sauce production in Southeast Asia has always employed slow bacterial fermentation with high salt concentration over prolonged periods of time.[63] *Lactobacillus sp.* are commonly utilized as a means of food preservation via fermentation. Lactic acid fermentation results in the production of lactic acid, acetic acid and other organic acids which reduced the pH and activate endogenous proteases (such as cathepsins) present in by-product tissues.[64] Reduced pH along with the antimicrobial chemicals (bacteriocins and antimicrobial peptides) produced by Lactobacilli inhibit the growth of other harmful bacteria.[65] Studies have demonstrated fish by-products subjected to lactic acid fermentation substantially reduced the populations of harmful microbes (*Staphylococcus sp*., *Clostridium sp*. and coliform bacteria) as compared to raw offal from processing.[66,67] The widespread application of this method across Asia is evidence of the potential for its use in other areas for human consumption.

Using microbes to activate proteases or acting as a source of proteases provides distinct advantages over commercial protease preparations. This is a significantly inexpensive option compared to enzymatic hydrolysis and the microbes can be cultured quickly for industrial processing. The strain of microbe used for proteolysis is based on their proteolytic potential, growth conditions and input requirements.[68] In contrast to typical industrial biotechnology applications, a sterile substrate is not required for the microbial processing of fish by-products. For instance, microbial population dynamics and product quality of fermentations of non-sterilized media with *Lactobacillus casei* were similar to those obtained for sterilized media, and pH stable fermentate was obtained within 72 hours.[69] Microbial fermentation processing without pre-

sterilization is also expected to be applicable within the framework of Good Manufacturing Practice (GMP) and Hazard Analysis and Critical Control Point (HACCP) for commercial production of fish protein hydrolysates.[70] In this regard, there are several traditional Asian food-products available currently, that utilize bacterial fermentation of unsterilized protein-based substrates.

Based on the proteolytic machinery involved, 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 and process parameters. 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.[48] Fermentation processes enhance preservation, organoleptic, textural and flavor properties,[71] whereas enzymatic hydrolysis typically leads to bitterness (as described in the previous section).

Factors affecting recovery of protein hydrolysate using microbial processing are similar to the parameters involved in enzymatic processing. Recovery is influenced by substrate tissue type, type of inoculum, amount of inoculum, processing duration, temperature, pH and agitation.[38] The broad range in protein yields from by-products is dependent on the bioprocessing approach along with the substrate properties. Lactic acid fermentation of shrimp wastes yielded protein 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* achieved a high recovery of up to 81% protein content (30% DH) in the hydrolysates.[26] In contrast, 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 muscle derived hydrolysates was higher as

compared to by-product cod hydrolysate (inherently lower abundance of protein in the starting material). Therefore, the protein recovery also depends on the tissue type and initial protein content of the substrate.[73] Although comparing the protein recovery of different studies, in terms of the impact of DH, to derive meaningful conclusions is not an ideal approach since different studies use different approaches to calculate both protein recovery and DH. Consistent and reliable analytical approaches (described in the next section) play a major role in being able to ensure data from within the field of research is comparable.

1.5. Analytical approaches to characterize peptide products

Protein hydrolysate and peptide fractions are composed of a mixture of diverse set of molecules. Different analytical approaches offer a window into better understanding this diversity and their behavior (functionality or physicochemical properties). A combination of various approaches can therefore provide an elaborate perspective on hydrolysate composition, properties, and bioactivity.

1.5.1. Amino acid content

The amino acid composition is the number of amino acids of each type normalized with the total number of residues. This approach typically relies on acid hydrolysis into constituent free amino acids which are derivatized (pre/post column) and separated based on reversed-phase liquid chromatography (described in section 1.7.2). Generally, the nutritional quality of protein extracts is evaluated based on the absolute content of essential amino acids, the relative proportions of essential amino acids to non-essential amino acid residues. Therefore, several studies utilize this amino acid analysis as a quality control parameter for protein hydrolysate production, especially from a nutritional perspective.[30,36,57] Amino acid content can be used for elucidating functional properties of peptides (QSAR (quantitative structure-activity relationship) modelling) based on the

abundance of hydrophobic (L, I, V, Y, F), anionic (E, D) or cationic (R, K) amino acid residues.[74,75] For instance, higher abundance of histidine is associated with higher ability of peptides to bind metal ions.[76] Therefore, amino acid content offers a unique perspective into peptide composition and properties.

1.5.2. Reversed-phase liquid chromatography

Separation based on polarity is the most predominantly used approach in the literature to generate peptide profiles. In this approach, a peptide mixture is introduced to a liquid chromatographic system (LC) with a C-18 column as the stationary phase and separated on the basis of polarity with a water-acetonitrile gradient as mobile phase. The elution profile can be used to understand the relative hydrophobicity of the component peptides in the sample. Doyen *et al*. (2013) used C-18 LC-UV (LC with a variable wavelength detector) profile to determine the migration rate of peptides during electrodialytic fractionation of a protein hydrolysate.[77] In addition to LC-UV peaks which are more commonly used to evaluate protein hydrolysates, LC-MS/MS total ion chromatograms can also be used to peptide profile of a mixture (protein hydrolysate). Therefore, a HPLC chromatogram generated with a reversed-phase separation can be used to evaluate impact of processing and monitor changes in the peptide components.

1.5.3. Size exclusion chromatography

With size exclusion chromatography (SEC), peptides are separated from largest to smallest in proportion to their molecular size in solution. Larger peptides are excluded from taking the paths in the packed bed and elute first, in the void volume. Smaller peptides penetrate and pass through the pores to various degrees that depends on their size, with the smallest molecules diffusing furthest into the pore structure of the stationary phase and eluting last. SEC is especially useful to determine the molecular weight distribution of a protein hydrolysate. Appropriate fractionation of

the protein hydrolysate using membrane filtration with a given molecular weight cutoff can be carried out based on the peptide molecular weight distribution of the protein hydrolysate.[80] This chromatographic approach while not used extensively, has more potential to be implemented by the natural bioactive peptide research community to monitor changes in peptide profile following fractionation/purification.

1.5.4. Ion exchange chromatography

Ion exchange chromatography utilizes differences in charge to facilitate the separation of peptides in a sample. Strong cation exchange (SCX) and weak anion exchange (WAX) chromatography are the most suitable for ion exchange chromatography of peptides. The sample is loaded in a low ionic strength mobile phase and eluted by increasing the ionic strength of the mobile phase.[78] Other approaches rely on altering the pH with or without changing the ionic strength to facilitate the elution of peptides.[79] Chromatographic peaks with longer retention times indicate the levels of higher charge state of the peptides or chain length. In SCX, an increase in pH can decrease the net positive charge of peptides leading to weaker binding and shorter retention times. Organic modifiers such as acetonitrile or methanol also affect retention of peptides by reducing hydrophobic interactions and improving electrostatic interactions with stationary phase.[79] There is potential for improved use of ion exchange separation as a means of profiling the charge distribution of peptide components in fractions and understanding the influence of the same on bioactivity.

1.5.5. Tandem mass spectrometry peptide sequencing

Tandem mass spectrometric analysis facilitates large-scale sequencing approaches such as proteomics and peptidomics for extensive characterization of a given sample. Protein identification is typically carried out using shotgun or bottom-up proteomics. In this approach, peptides are

generated through enzymatic hydrolysis of the sample and are separated by reversed-phase liquid chromatography followed by spectral matching comparison with *in silico* peptides generated from the specific protein database. Peptide sequences can be used to determine an array of information about the sample components such as molecular weight distribution, grand average of hydropathy (GRAVY) scores (a measure of hydrophobicity),[80] and isoelectric points (pI, indicative of peptide charge).[81] Identified peptide sequences can also be used with *in silico* tools to predict biological activities using bioactive peptide databases,[82] and identification of peptide-protein interactions.[83]

Identifying peptides generated by non-specific cleavages from commercial protease preparations or autolytic approaches relies on an unspecified cleavage database search i.e., a search without any pre-defined cleavage pattern. In this approach, every potential cleavage site or amino acid residue is considered for developing fragment ion lists from protein sequence databases. This results in a much broader search space compared to the more prevalent approach of using tryptic hydrolysis. The probability of incorrect identifications is enhanced with this approach thereby reducing identifications. MS peptide sequencing approaches are associated with some inherent biases such as peptide ionization efficiency and mass range. Generally, peptide identifications using tandem mass spectrometry are carried out between *m/z* 400-1600. While tandem mass spectrometry offers a large-scale analysis for identification and quantification of peptide components in a sample, there are significant biases associated with the detection and identification using this approach.

1.5.6. In vitro assays: antioxidant and other characteristics

Oxidation is an essential reaction in all living organisms, as the formation of free radicals and other reactive oxygen species (ROS) plays an important role in signal transduction. Oxidative stress occurs from the imbalance between production of excess ROS and the cellular antioxidant defense

system. This is detrimental to tissue and can induce damage to other cellular components in lipids, carbohydrates, proteins and nucleic acids. Consequences of oxidative stress in this organ include mitochondrial dysfunction, altered neuronal signaling and inhibition of neurogenesis, and can contribute towards heart disease, stroke, arteriosclerosis, diabetes, and cancer. Peptide antioxidants can exhibit antioxidant activity by several different mechanisms such as inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and alteration of the physical properties of food systems.[84,85] Assays monitors the ability of peptides to act as an antioxidant based on these mechanisms. The ferric reducing capacity is determined by the ability of antioxidant peptides to reduce ferricyanide to ferrocyanide, which binds to free Fe^{3+} ions to forms Prussian blue. In the literature, peptide ferric reducing antioxidant potential is described based on glutathione (GSH), a natural regulator of redox potential in living systems and is expressed as GSH equivalent.[86] A metal chelation assay evaluates the ability of peptides to chelate ferrous ions from $FeCl_2$ competing with FerroZine™ (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid) which produces a blue color on binding Fe^{2+} ions.[87] Peptide chelation capacity is expressed as ethylenediaminetetraacetic acid (EDTA) equivalent or % of Fe^{2+} ions bound. Additionally, there are several assays that monitors the ability of peptides to quench free radicals (sacrificial antioxidant ability) such as hydroxyl, DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid). The ability of peptides to inhibit dipeptidyl peptidase 4 (DPP4) is linked towards hypoglycemic and immunomodulatory activities.[88] Similarly, the ability to inhibit angiotensin-converting enzyme (ACE) and renin have been used in literature to evaluate antihypertensive activity of peptides.[89] These *in vitro* assays are a quick and easy way to evaluate and screen potential of food-derived bioactives for possible bioactivity *in vivo*.

1.6. Functionality of fish protein hydrolysates

Physicochemical properties such as charge, size, solubility and hydrophobicity influence the biological activities of food-derived peptides. For instance, Szeto (2006) described that cationic peptides with higher hydrophobicity translocate into the inner mitochondrial membrane and induce antioxidant activity within the intracellular system.[90] Zou *et al*. extensively reviewed the structural features of proteins and peptides that underlie *in vitro* antioxidant properties.[74] Smaller sized peptides have been typically associated with higher reducing capacity. For instance, a comparison of different proteases for hydrolysis of tilapia (*Oreochromis niloticus*) frame protein found that the tryptic hydrolysate demonstrated the highest DH (therefore, the smallest peptides) and antioxidant activity.[91] The cleavage specificity can also play a major role in peptide functionality. A study conducted by the group at the Verschuren Centre demonstrated enhanced Fe (pro-oxidant metal) chelation capacity of Flavourzyme salmon hydrolysates as compared to hydrolysates generated from microbial fermentation or formic acid treatment of the same Atlantic salmon substrate.[8] There is growing evidence towards associating peptide properties such as molecular weight and presence of hydrophobic/aromatic amino acid residues with higher antioxidant activity. However, antioxidant activity via higher radical scavenging has been typically exhibited by high molecular weight peptide fractions, which can contribute to lowering lipid peroxidation.[92] In addition to aqueous peptide extracts, other formulations such as emulsion can also have significant functional benefits. For instance, Belhaj *et al*. (2013) demonstrated that phospholipopeptidic complex (emulsion) 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.[93] As mentioned in section 1.5.6, peptides can exhibit antioxidant activities in different forms (reducing, metal chelation and radical scavenging). Each of these mechanisms of antioxidant

activity is impacted differently due to peptide properties such as molecular weight, composition, polarity and charge.

Microbial processing has relevance towards novel product development with environmentally sustainable and economically feasible approaches. *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.[94] In addition, another study comparing acid and fermentative hydrolysis found the fermentative-hydrolysate to exhibit higher radical (hydroxyl, ABTS, DPPH) scavenging activity and antagonistic activity against *Salmonella typhi*.[95] DH was also demonstrated to exert a considerable positive influence on antibacterial activity of trout (*Oncorhynchus mykiss*) by-product hydrolysate prepared with trout pepsin.[96] Comparable antioxidant activity and enhanced antibacterial activity (against *Listeria monocytogenes*) were observed in fermentative and enzymatic protein hydrolysate.[97] As with other processing approaches, emulsifying and foaming properties of peptides are governed by the size of peptides and the concentration employed.

1.7. Pretreatment, modification and incorporation into food matrices: Bioactive peptides

In addition to protein hydrolysis, physical treatments or pretreatments are carried out to improve recovery. Physical pretreatment of protein concentrate or tissue homogenate has been utilized in several studies.[98–100] Ultrasound treatment incorporated processing of tilapia (*Oreochromis niloticus*) muscle protein resulted in higher reducing capacity and improved radical scavenging activity.[101] Another approach is the application of microwave pretreatment which improved trout frame protein DH, protein solubility and recovery, along with improved antioxidant activity.[102,103] Pre-treatments with physical methods listed above can result in the functional modifications of proteins/peptides, influencing their secondary, tertiary and quaternary

structure.[104,105] Modifications can arise as a consequence of processing or treatments.[49,62] Chemical modifications can also be induced on protein hydrolysates to impart desired functionalities. Certain pretreatments can result in the formation of conjugates such as the Maillard reaction products resulting from the glycosylation of proteins and peptides.[106] For instance, Djellouli *et al*., (2019) observed enhanced ferric reducing capacity (22-fold), scavenging radical (7-fold) and metal chelating (7-fold) activities along with antibacterial activity against gram positive and gram-negative bacteria as a result of conjugation of glucosamine to shrimp protein hydrolysate.[107]

Bioactive peptides incorporated into a food matrix can contribute to preservation by scavenging free radicals, interacting with metal ions, inhibiting lipid peroxidation; they can also inhibit the growth of microorganisms. These functional properties in addition to their *in vivo* biocompatibility put bioactive peptides in prominence as a promising alternative to chemical preservatives. For instance, pollock protein hydrolysate applied as a glaze on salmon fillets have been shown to be effective in improving storage quality and limited lipid peroxidation.[108] Incorporation of gelatin hydrolysates were found to negatively impact textural qualities and acidification in low fat and fat free yogurts.[109,110] In contrast, Gheslaghi found that better gelatin hydrolysate incorporation improved solid-like behavior and elastic gel structure in fat-free set-type yogurt.[111] Fish protein hydrolysate when incorporated into soup was found to have sensory acceptability up to a concentration of 3% without traces of bitterness.[112] Fish protein hydrolysate incorporation into yogurt and storage over a period of 7 days under refrigeration did not change its antioxidant activity and ACE-inhibitory activity.[109] Encapsulation of peptides by liposomes, protein micro/nanoparticles or other means can improve their stability and bioavailability *in vivo*.[113] It is important to note that surface properties (charge, hydrophobicity) of peptides do play a role in determining the encapsulation properties.[114] The processing conditions or techniques

plays a significant role in influencing the conformation, post-translational modifications, and other physicochemical properties of bioactive peptides.

1.8. Storage stability of marine peptide formulations

An understanding of storage stability and behavior is essential to peptide bioproduct formulation since it impacts processing steps such as fractionation, purification and packaging, along with consideration for steps in the supply chain such as storage conditions and shelf life. Storage stability, in terms of chemical stability of fish protein hydrolysates have been extensively reviewed by Rao *et al*., (2016).[115] Chemical changes that occur during storage can lead to modified structure, appearance, texture, sensory properties and functionality of protein hydrolysates. Klompong *et al*., (2012) found that fish protein hydrolysate had higher browning intensity as a result of storage.[116] Generally, several reports indicate that longer storage of fish protein hydrolysate resulted in color change (browning intensity), change in pH, production of 2-thiobarbituric acid reactive substances (TBARS; lipid peroxidation products) and reduction in solubility.[116–118] Benjakul and Morrissey postulated that oxidation of myoglobin or melanin results in the darker color for hydrolysates during storage.[119] Fish protein hydrolysates are reported to have higher levels of lipid peroxidation products as a result higher fat content in the matrix.[117,120] The low solubility arises as a result of aggregate formation during storage. Zhou *et al*., (2008) demonstrated that the formation of disulfide linkages is responsible for ~90% of the aqueous insoluble aggregates formed during storage of whey proteins.[121] The functionality of fish protein hydrolysates is dictated by their physicochemical properties. Thiansilakul *et al*., (2007) did not observe significant changes in ferric reducing capacity of round scale protein hydrolysates over a period of six weeks at both 4 °C and 25 °C.[122] In the same study, the solubility of hydrolysates over the storage period was also evaluated without any significant differences.[122]

1.9. Fractionation of fish protein hydrolysates

Fractionation is necessary to enrich select peptides of a given nature that can potentially enhance functional activities. This is of relevance from the perspective of the abundance of these peptide ingredients in the final product for consumption. Additionally, the amount of bioactive peptides that eventually reach a target tissue typically tend to be low as well, which also affects the bioavailability. Generally, smaller sized peptides (<10 kDa) recovered from protein hydrolysates have been reported to exhibit higher biological activities. Smaller peptides can be obtained either by manipulating the degree of hydrolysis (DH) during enzymatic hydrolysis or by fractionation. However, industrial applications of bioactive peptides have been severely limited by cost of production, product stability and application on a commercial scale. Enrichment or fractionation of peptides using scalable downstream technologies is one approach to overcome some of these barriers. There is growing interest in the food industry towards the development of continuous downstream processing approaches for large-scale production of bioactive molecules from sustainable sources. Separation of heterogenous mixtures of peptides in fish protein hydrolysates is usually carried out to obtain fractions of interest and enhance the value of the product. Several studies have relied on chromatographic approaches for the separation of proteins or peptides following processing (Reviewed by Kitts and Wheeler, 2003).[123] Chromatographic approaches, however, are not scalable to commercial applications. Currently, membrane technology is being employed for large scale protein concentrate preparation, especially in the dairy industry, and even for marine sources.[124,125] Application of nano/ultrafiltration approaches has been extensively reviewed for recovery of proteins from seafood processing waters.[126] Filtration approaches are reliable and easily scalable for commercial production. However, these approaches are limited by low selectivity and fouling (with complex substrates). For instance, a

bioreactor coupled to an ultrafiltration unit has been demonstrated to facilitate protein recovery from Sardine waste at pilot scale (60 L) utilizing crude peptic enzyme extracts.[53] 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.[125] An extensive review of separation strategies, especially pertaining to membrane processing for food-based peptide bioproduct formulations, is presented in Chapter 4.

Protein isolation and enzymatic processing costs have limited the commercial production and application of peptide bioproducts in the food industry. Novel scalable processing approaches such as microbial fermentation along with commercially viable fractionation techniques need further advancement for developing functional and viable protein hydrolysates/peptide bioproducts. Development of effective techniques for concentrate preparation, large-scale hydrolysis and peptide recovery with improved functional properties can lead to the sustainable and economical production of protein hydrolysates.

1.10. Objectives of the present study

Reports on marine by-products sources used for extraction of bioactive peptides generally tend to have elaborate, multi-stage processing steps that rely on distinct protein isolation and hydrolysis steps. At the Verschuren Centre, a simplified, scalable and integrated processing approach has previously been developed for the production of by-product salmon protein hydrolysate.[7,38] Figure 1.2 depicts the steps involved in the protein hydrolysate preparation using this approach. In contrast to the process described in Figure 1.1, this approach involves two stages: (i) autolysis induced by lactic acid bacterial fermentation followed by ii) centrifugal separation of the protein hydrolysate. Fermentative processing used here results in the production of a unique fish protein hydrolysate derived from Atlantic salmon by-products.

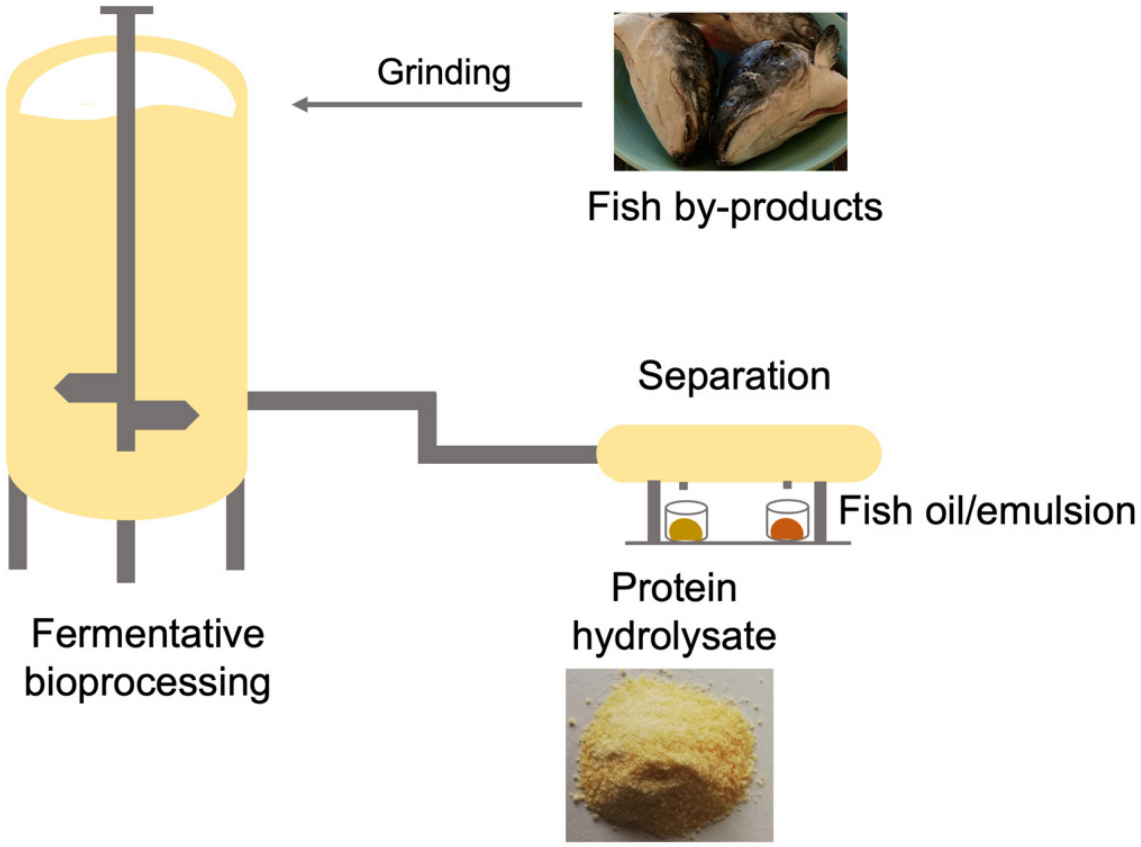

Figure 1.2: *Illustration of process for the fermentative production of fish protein hydrolysate*

The bioprocessing results in the generation of the protein hydrolysate fraction, along with other fractions, namely emulsion, oil and residue. The emulsion fraction, especially, has not been well-characterized in the literature. The assessment of the physicochemical characteristics and functionality of this lipopeptidic complex is necessary for directing this product stream to an appropriate application. Therefore, in the second chapter the primary objective is to evaluate and characterize the protein hydrolysate fraction in contrast to the emulsion fraction. This ensures the workflow development in the form of a biorefinery approach, which is essential for maximum resource utilization as part of the by-product upcycling process. Moreover, to further refine, enrich

and improve the biological activities of the hydrolysate generated from the salmon by-products, scalable, cost-effective and selective fractionation approaches need to be developed. To this end, further characterization will also ascertain the viability and stability of the protein hydrolysate for storage and downstream processing.

Atlantic salmon (*Salmo salar*) by-product hydrolysate fractionation using a scalable approach will have the potential to facilitate development of a wide array of peptide products with unique physiochemical and functional activities. Separation and purification enhance the potential of these co-product streams for nutraceutical and ingredient development. Different techniques used in the production and downstream processing of protein hydrolysates can facilitate bioproduct development with predetermined properties positively influencing functionality. The present study aims to develop a membrane processing approach to enable the capacity to selectively separate components of a peptide mixture based on desired attributes such as charge and molecular weight. In this regard, electro-ultrafiltration is a suitable separation approach and combines the electrophoretic separation of charged moieties with conventional membrane filtration. A major objective of the present this study is the assessment of optimal parameters for electro-ultrafiltration of Atlantic salmon protein hydrolysate. A model BSA protein hydrolysate is used to evaluate the selectivity of the electro-ultrafiltration using LC-MS/MS characterization, prior to using the complex salmon protein hydrolysate. Improved selectivity is expected to be achieved through the modification of the filtration conditions such as ionic strength, pH, sample concentration, applied pressure, electric potential and solvent composition. Following this, fractionation of salmon protein hydrolysate will be carried out to enrich peptides of desired physicochemical properties. Assessment of filtration performance (selectivity and recovery) during fractionation with electro-ultrafiltration is carried out using various analytical strategies. The solute behavior during

fractionation can contribute towards the enhanced understanding of separation science of peptide behavior within a complex unique matrix (protein hydrolysate derived from fermentation) during fractionation. Peptide composition/diversity is to be evaluated in the various fractions. Functional properties of peptide fractions is assessed to determine the impact of fractionation on bioactivity. This can also help expand the knowledge on peptide properties that contribute to different functionalities. The present study is part of an ongoing research initiative at the Verschuren Centre to develop processes and bioproducts that valorize and facilitate the full utilization of marine by-products. By-products from Atlantic salmon (*Salmo salar*) is especially relevant as a major commercial aquaculture species in Atlantic Canada and around the world.

CHAPTER 2

Characterization of protein hydrolysate and emulsion fractions derived from bioprocessed Atlantic salmon by-products

Abstract

Sustainable utilization of fish by-products is a major step towards value addition in aquaculture production. Microbial fermentation presents itself as a scalable and sustainable approach to extract high value products from fish processing co-products. Centrifugal separation of the by-products of Atlantic Salmon was shown to yield four distinct fractions: the oil, residue, emulsion and aqueous hydrolysate. The oil fraction is comprised exclusively of lipids, whereas the residue fraction consists of bone fragments along with minerals, unhydrolyzed tissue and other insoluble material. As demonstrated here, the emulsion fraction is a heterogenous mixture of proteins/peptides (24%) and oil (60%). While the oil and emulsion fraction have a similar fatty acid profile, the peptides vary significantly between the emulsion and aqueous protein hydrolysate fractions in their physicochemical properties (molecular weight, amino acid composition, hydrophobicity). This study also indicated that peptide hydrophobic interactions facilitated the formation of the emulsion fraction. Both hydrolysate and emulsion peptides fractions were observed to have comparable ferric reducing antioxidant potential, while Fe(II) chelation capacity was also found to be higher in the emulsion (98 ± 1 μM EDTA equivalent) compared to the hydrolysate (28 ± 8 μM EDTA equivalent). These findings are relevant to our understanding of the distinct characteristics of peptides as fish by-products undergo fermentative processing and separation. Additionally, the results contribute towards novel product development from microbial

processing of fish by-products such as the application of hydrolysate and emulsion fractions in food formulations.

2.1. Introduction

Fish processing by-products have traditionally been recognized as low-value waste-streams with negligible market value beyond their use as animal feed or fertilizer. Additionally, the disposal of these waste streams is both expensive and a major cause of environmental pollution.[127] Fish processing co-products are reservoirs for bioactives such as omega-3-fatty acids contained in fish oil. Additionally, considering that large amounts of food-grade proteins are present in fish waste materials, proteolysis-based processes are being developed to recover these proteinaceous materials. In doing so, under-utilized fish and fish by-products could potentially be converted into higher value ingredients within the food industry (food ingredients,[128] nutraceuticals,[129] functional foods[130] or natural preservatives).[131]

In addition to the nutritional value, food derived peptides are used to prepare nutraceuticals (encapsulated fish oil, glucosamine pills etc.) and as ingredients for functional food (health drinks, fortified cheese, yogurt etc.). Generation of functional foods and nutraceuticals from fish by-products has inherent economic and environmental advantages, in addition to the significant human health prospects. The generation of aqueous protein extracts from Atlantic Salmon and other marine sources is well described in the literature.[112,132–134] The recovery, nutritional and functional properties of salmon proteins can be tailored by proteolysis under controlled conditions.[18,43,51,56,93,135–137] Enzymatic,[30] bacterial[136] and chemical[95] 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.[27,62]

The physicochemical characteristics of the hydrolyzed protein directly affect their functional properties,[42] yield[24] and use.[138]

The fermentative bioprocess developed previously at the Verschuren Centre, used in this study, is unique in that neither a protein isolation nor extraction step is carried out prior to hydrolysis, which results in the formation of the multiple fractions. The bioprocessed salmon by-product mix resulted in the expected four distinct fractions (oil, emulsion, hydrolysate and residue) following centrifugation.[8] A biorefinery approach to utilizing the multiple fractions is necessary to ensure these resources are used to their full potential. In the present study, the different fractions generated from salmon by-product fermentation were characterized. As a result of its potential for widespread applicability, emphasis will be placed on the hydrolysate fraction along with the emulsion fraction (which has not been described well in the literature). The findings from this research are expected to contribute towards the understanding of the formation of these different fractions, particularly pertaining to the behavior of component peptides.

2.2. Materials and methods

Atlantic salmon processing by-products consisting of heads, frames, skins and viscera were collected in Styrofoam boxes on ice from a salmon aquaculture processing plant in Atlantic Canada. The by-products were shipped overnight to the Verschuren Centre Inc., Sydney, NS, Canada, and immediately stored at -20 ºC prior to processing. The modified Lowry assay kit, and HPLC grade solvents were from ThermoFisher Scientific (Ottawa, Canada). Water was purified with a Sartorius Arium® Advance and then passed through a Barnsted nanopure system (ThermoFisher Scientific (Ottawa, Canada)).

2.2.1. Lactic acid bacterial fermentation and centrifugal separation

Salmon by-products were ground to a slurry (~1 kg) and mixed with 5% (w/w) deproteinized whey powder and 1% (w/w) bacterial inoculum in a fermentation flask.[8] The mixtures were incubated at 37 °C for 48 hours in an Eppendorf Bioreactor with continuous agitation at 300 rpm. After fermentation, samples were transferred into weighted falcon tubes and fractions were obtained by centrifuging at 4000×g for 20 min. Additionally, the collected emulsion and hydrolysate fractions were lyophilized (Labconco, MO, US) prior to further analysis.

2.2.2. Proximate composition of emulsion fractions

The proximate composition of all fractions was analyzed. The moisture content of emulsions was determined by oven-drying the sample at 105 °C for 24 h. Ash represents the incombustible mineral components remaining after degradation of organic matter in the sample. The ash was determined by heating the sample (~1 g) in a Muffle furnace in a crucible at 500°C for four hours (AOAC, 1990). The mass of the residue remaining is expressed as a percentage of the initial starting material. Crude lipid/fat content of dried sample was analyzed by gravimetry following a petroleum ether extraction using Soxtherm (Gerhardt Analytical Systems, Germany). The total nitrogen content of dried samples was measured using an elemental analyzer (2400 CHNS/O Series II System, Perkin Elmer, USA). The crude protein content was then calculated using the formula as follows:

Protein content (%) = total nitrogen (%) × F, … (2.1)

where the conversion factor F is assigned a value of 6.25.[139]

2.2.3. Lipid analysis

The fatty acid compositions was determined by extraction in the presence of internal standards, tridecanoin for total lipid/fatty acid analyses and diheptadecanoyl phosphatidylcholine

(NuChek Prep, Elysian, MN, USA) for phospholipid analyses (modified from Bligh and Dyer).[140] An aliquot of the total lipid extract (lower phase) was taken for quantitation of the fatty acids following transmethylation and another aliquot was taken for separation of the triglyceride and total phospholipid via thin-layer chromatography prior to transmethylation of these fractions. Methyl esters were prepared with boron trichloride in 12% methanol and heating the methylation tubes at 95 °C for 30 minutes. The fatty acid methyl esters were analyzed on an Agilent 7890B GC-FID with a 60 m DB-23 capillary column (0.32 mm internal diameter) using a standard mixture (qualitative and quantitative) with the known fatty acid components for verifications (from Nu Chek Prep, Elysian, MN, USA).

2.2.4. Emulsion defatting

Defatting was carried out by suspending the dried sample in diethyl ether at a ratio of 1:20, (ThermoFisher Scientific) followed by centrifugation (4500×g, 15 min). The process was repeated twice and sample air dried.

2.2.5. Gel permeation chromatography analysis

Defatted samples (4 mg/mL) were injected (20 µL) to a Polysep-GFC-P2000 column (Phenomenex, CA, USA) connected to an Agilent 1260 Infinity series II LC equipped with a variable wavelength detector (VWD) detector set at 214 nm. The mobile phase at flow rate of 0.85 mL/min was composed of 45% acetonitrile and 0.1 % TFA in nanopure water. The calibration curve was prepared using cytochrome C (12327 Da), bovine insulin (5733 Da), angiotensin (1046 Da) and glutathione (307 Da).

2.2.6. Evaluation of peptide mass ranges

Peptide recoveries were carried out on a 1 mm × 50 mm self-packed reversed phase column containing POROS® R2 beads (Applied Biosystems, Carlsbad, CA).[141] The purified peptide

fractions of emulsion and hydrolysate collected in a fraction collector were dried and resuspended in 0.1% formic acid in water to equivalent amounts. Two replicate injections (10 µL) of the sample were separated on self-packed column containing, 4 µm C12 beads (Phenomenex)) within a nanospray tip (New Objective, Woburn, MA). A dual capillary LC system (Agilent) was interfaced to an LTQ linear ion trap mass spectrometer (ThermoFisher Scientific, San Jose, CA).[142] The LTQ operated in data-dependent mode (MS followed by MS/MS of top three peaks) with 30 s dynamic exclusion. Lists of unique peptide mass ranges were obtained as a multiconsensus of 2 repeat injections using Proteome Discoverer v1.4 (ThermoFisher Scientific, San Jose, CA) comparing with the *Salmo salar* UniProtKB database (downloaded June 2021, 82272 entries).

2.2.7. LC-MS/MS peptidomic analysis

Defatted emulsion and hydrolysate fractions were desalted using ThermoFisher Scientific™ HyperSep™ C18 Cartridges and dried. The LC-MS/MS analysis of resuspended samples was carried out at SPARC Biocentre (ON, Canada) with a Q Exactive HF-X (ThermoFisher Scientific, San Jose, CA) operating at a resolution of 60,000 at positive polarity with a scan range of *m/z* 400 to 1600 fitted to an Easy nLC system (ThermoFisher Scientific, San Jose, CA, USA). Reverse-phase separation of peptides was carried out (60 min) in the nLC running at 250 nL/min with a gradient program of 3% B at 0 min to 20% B at 18 min to 35% B at 49 min to 100% B at 51 min. Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile.

Peaks studio 10.6 (Bioinformatics solutions Inc., ON) was used for peptide searching against *Salmo salar* UniProtKB database (downloaded June 2021, 82272 entries) with parent mass error tolerance of 10 ppm and fragment mass error tolerance of 0.2 Da. Peptide lists were generated with database searches carried out using unspecified cleavage (no enzyme) with a peptide false

discovery rate of 1%. The emulsion and hydrolysate peptide lists were compared using Venny 2.1 (BioinfoGP). Isoelectric points of the identified peptides were calculated using isoelectric point calculator (http://ipc2.mimuw.edu.pl/).[81] GRAVY scores for the identified peptides were computed using an online tool: https://www.bioinformatics.org/sms2/protein_gravy.html.[7]

2.2.8. Proteomic analysis

The Atlantic salmon emulsion fraction (50 mg) was mixed with 2.5% (w/v) SDS in tris tricine buffer (100 mM of each component, pH 8.3). The solution was boiled for 5 min followed by 10 min of centrifugation (13,300 rpm, 10 min). The supernatant was diluted 5-fold and added to transmembrane electrophoresis[3] sample wells with tris tricine buffer (100 mM) in the buffer chambers. The device was set to run at constant current (100 mA) for 60 min. In contrast, the hydrolysate fraction was already soluble in water and did not require an extraction step. Trypsin digestion was carried out for both samples as described previously.[143] Desalted samples were analyzed on LC-MS/MS as described in section 2.2.7. Database searches were carried out with trypsin specified cleavage with Peaks studio 10.6 (Bioinformatics solutions Inc., ON) with the same parameters as described in section 2.2.7.

2.2.9. Amino acid content analysis

Defatted samples (0.0140 - 0.0160 g) were added to borosilicate tubes followed by the addition of 450 µL of 6 N HCl with 1% phenol. The borosilicate tubes were added to a PicoTag reaction vial (Eldex Laboratories Inc., Napa, CA, USA). The reaction vial was then treated under vacuum and heated (110 ºC) for 24 hours. Following hydrolysis, the samples were vacuum dried to remove residual HCl. Derivatization was performed with phenyl isothiocyanate (PITC, Sigma Aldrich, Oakville, ON). The derivatization reaction was carried out at room temperature using a 7:1:1:1 methanol, water, triethylamine, and PITC mixture. The derivatized samples were then

diluted to 250 µL using sodium phosphate (100 mM). In tandem with the derivatization of the samples, a standard solution with 16 amino acids was derivatized using the same procedure. Diluted sample/standard (2 µL) was injected on a Waters Acquity UPLC equipped with a UV detector (254 nm) and a flow rate of 0.5 mL/min. The column was a BEH C18, (1.7 µm particle size), 2.1 x 100 mm (Waters, ON, Canada). Reversed-phase gradient elution was achieved using a mixture of Buffer A (0.14 M sodium acetate, 0.05% triethylamine, pH 6.05, 6% acetonitrile) and Buffer B (60% acetonitrile in buffer A).

Based on the injected amount (50 picomoles) and integrated areas of each amino acid in the standard, the quantities in the unknown sample were determined. The total protein content in the derivatized sample was determined and expressed as a percentage of protein by mass.

2.2.10. Ferric reducing antioxidant potential assay

Reducing capacity of samples at 4 mg/mL was estimated as using the method developed by Oyaizu (1986)[144] with modifications from Mohan *et al*., (2015)[62] and expressed based on glutathione (GSH) mM equivalent.

2.2.11. Metal chelation assay

Metal chelation assay was performed based on a method developed by Boyer and McLeary (1987) and modified by Saidi *et al*., (2014).[87,145] The 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 at room temperature. The mixture (200 µL) was then transferred to a 96-well plate and the absorbance was measured at 562 nm using a microplate reader (Tecan M1000 microplate reader, Männedorf, Switzerland).

2.3. Results and Discussion

2.3.1. Processing and recovery of fractions

Lactic acid fermentation is used in this study as a cheap, sustainable and novel approach to release biomolecules from the fish by-product tissue and has been developed previously.[8] Although fermentation has been used traditionally in Eastern Asia to produce fish sauce. It was observed that the liquefaction of salmon tissues, which is the degradation of structural components of ground tissues resulting in the release of inherent moisture from the mix of ground by-products, was relatively fast, taking 1-2 hours of processing in the bioreactor. This is as a result of thermal and acidic activation of proteolytic enzymes following the production of lactic acid during bacterial fermentation which rapidly results in release of moisture, soluble components, and oil droplets from salmon tissue.[8] A previous study from the group at the Verschuren Centre has described the microbial processing of salmon viscera, wherein lactic acid bacteria (LAB) consume lactose to generate an acidic environment, triggering the activation of endogenous enzymes in the by-product tissues.[8] This facilitates the hydrolytic release of oil, peptides and other mineral components for subsequent downstream processing.[8] A key finding of this study was that the salmon fermentate separates into four distinct fractions, namely, the oil, emulsion, soluble (aqueous) hydrolysate and the residue phase (Figure 2.1).[8] While these fractions have been identified previously, their composition and basis of formation has not been described. The next section compares the proximate composition of these fractions.

Figure 2.1: *Centrifugally fractionated slurry of the bioprocessed salmon by-products*

2.3.2. Composition of fractions

The fractions generated by centrifugal separation were observed to have significant differences in their composition. The proximate composition of the hydrolysate, emulsion and residual fractions are summarized in Table 2.1**.** The aqueous protein hydrolysate is composed of a heterogenous mixture of aqueous components such peptides, minerals, and nucleic acids. As expected, the crude protein content is highest in the aqueous protein hydrolysate (58 ± 5%) and is significantly higher ($p<0.05$) compared to the emulsion fraction (28 ± 5%). The protein content in a commercial hydrolysate product (Peptidyss®; prepared from sardine by-products via enzymatic hydrolysis) was reported to be >74%,[146] which is far greater than the 58% observed here. Likewise, the ash (mineral) content in the salmon protein hydrolysate fraction (10.2 ± 2%) was found to be

low compared to Peptidyss® (~20%).[146] The mineral content was even lower in the emulsion fraction (3.6 +/- 1%). Ca, Mg, Na, and Zn contents were much higher in the emulsion fraction, than previously described by Belhaj *et al*.[93] The phospholipopeptidic fraction as described by Belhaj *et al*., (2013) was generated by enzymatic hydrolysis of salmon heads.[93] It was described in the above-mentioned study that that the lipopeptidic complex is formed from enzymatic hydrolysis with a low degree of hydrolysis (therefore containing larger peptides). In contrast, previous studies from the group at the Verschuren Centre, have reported consistent formation of emulsion even with a high degree of hydrolysis (40-50%) through bacterial fermentation of salmon by-products.[8,92] As expected, the emulsion fraction has significantly ($p<0.05$) higher fat content (55 ± 8%) compared to both the aqueous hydrolysate fraction as well as the residue fraction. Therefore, the emulsion layer formed as a sandwiched layer between the oil and aqueous layers. It is largely composed of emulsified oil along with other lower density materials such as proteins that can interact with oil. The fraction is expected to be formed during the distribution of molecular components under the influence of the centrifugal force during separation. Comparison of lipid composition (emulsion and oil) and peptide characteristics (emulsion and hydrolysate) is described in the following sections to evaluate their contribution to the formation of the emulsion layer.

The residue is the bottom layer after centrifugation, largely composed of bone and other heavier and insoluble sediments (intact tissue, insoluble proteins), formed following the centrifugation of processed fish tissues. The presence of bone fragments and larger particles made sampling of the residue fraction challenging, resulting in high variability. The variability contributes to the lack of significance, when comparing the means.

***Table 2.1**: Proximate composition of fractions expressed as % of dry matter content.*

	Salmon fractions (mean ± std. dev.)		
	Hydrolysate	Emulsion	Residue
Crude Protein %	58 ± 5*	28 ± 5	54 ± 5*
Oil %	0.7 ± 0.5	55 ± 8*	9 ± 4
Ash %	10 ± 2	4 ± 1	15 ± 10
Calcium %	1.3 ± 0.2	0.9 ± 0.1	4 ± 4
Phosphorus %	1.6 ± 0.2	0.7 ± 0.1	3 ± 2
Sodium %	1.1 ± 0.2*	0.38 ± 0.04	0.75 ± 0.04*
Magnesium %	0.15 ± 0.02*	0.06 ± 0.01	0.13 ± 0.02*
Zinc (ppm)	470 ± 200	100 ± 10	590 ± 500

*indicates a statistical significance of $p<0.05$ in comparison of means using one way analysis of variance

2.3.3. Lipid profile

Belhaj *et al*. (2013) found that the lipid composition of salmon head emulsion complex consisted of 65% polar lipids and 35% triacylglycerols. In contrast, in the present work, it was observed that both the oil and emulsion fraction had significantly ($p<0.05$) lower abundance of phospholipids (22.3 ± 1 and 19.4 ± 6 g/kg of sample) respectively. The total fatty acid profile of the emulsion and oil fraction are presented in Table 2.2. The fatty acid profiles of two fractions were observed to be identical, with no statistically significant difference ($p<0.05$). The omega-3 fatty acid content was observed to be lower than values reported previously.[93] The fatty acid profile is dependent on the diet, time of year, feed and origin of the salmon. The lipid components are not

predicted to have contributed towards the formation of the emulsion fraction since the lipid composition is identical between the oil and emulsion fraction.

Table 2.2: *Fatty acid composition of emulsion and oil fractions derived from LAB processed salmon by-products.*

	Fatty acid content in fraction	
Fatty Acid	Emulsion (%)	Oil (%)
C10:0	0.011 ± 0.002	0.005 ± 0.001
C12:0	0.04 ± 0.03	0.020 ± 0.002
C14:0	1.93 ± 0.08	1.9 ± 0.1
C14:1	0.0790 ± 0.0003	0.080 ± 0.001
C16:0	14.6 ± 0.4	14 ± 0.5
C16:1n7	5.2 ± 0.1	5.2 ± 0.1
C18:0	4.080 ± 0.004	3.9 ± 0.1
C18:1	42.2 ± 0.6	42 ± 1
C18:2N6	16.6 ± 0.5	17 ± 1
C18:3N6	0.37 ± 0.04	0.38 ± 0.05
C18:3N3	2.3 ± 0.2	2.4 ± 0.1
C18:4N3	0.46 ± 0.04	0.49 ± 0.05
C20:0	0.17 ± 0.01	0.17 ± 0.01
C20:1	2.2 ± 0.2	2.19 ± 0.2
C20:2N6	1.06 ± 0.9	1.09 ± 0.10
C20:3N6	0.52 ± 0.02	0.550 ± 0.001
C20:4N6	0.53 ± 0.08	0.55 ± 0.1
C20:3N3	0.20 ± 0.03	0.20 ± 0.03
C20:4N3	0.40 ± 0.1	0.42 ± 0.1
C20:5N3	2.2 ± 0.5	2.3 ± 0.6
C22:0	0.11 ± 0.03	0.10 ± 0.03
C22:1	1.02 ± 0.5	0.97 ± 0.4
C21:5N3	0.13 ± 0.03	0.15 ± 0.01
C22:2N6	0.15 ± 0.04	0.15 ± 0.04
C22:4N6	0.13 ± 0.01	0.15 ± 0.01
C22:5N6	0.90 ± 0.01	0.10 ± 0.02
C22:5N3	0.91 ± 0.2	0.96 ± 0.2
C22:6N3	2.2 ± 0.6	2.4 ± 0.7
C24:0	0.02 ± 0.02	0.02 ± 0.003
C24:1	0.150 ± 0.005	0.140 ± 0.001
Total (mg/g of sample)	488 ± 93	854 ± 83
Phospholipid content (mg/g of sample)	22 ± 1	19 ± 6

2.3.4. Peptide profile

As mentioned in the previous section, Belhaj *et al*., (2013) postulated that the formation of the emulsion fraction (phospholipopeptidic complex) to be a result of larger molecular weight peptides from a lower degree of hydrolysis.[93] The study reported a high abundance of peptides between the molecular weight ranges 4 - 13 kDa. Generally, the emulsifying ability of larger molecular weight peptides is via their structural flexibility that enables peptide interaction with oil droplets while also associating with water. Peptide molecular weight as determined by the GPC analysis does not show a statistically significant difference in the abundance of larger molecular weight peptides in the two fractions. Table 2.3 depicts the peptide molecular weight profile of the emulsion and hydrolysate fraction. In contrast to the presented results, marine peptides (oyster) prepared by 4 hours of enzymatic hydrolysis were found to have approximately 71% of the sample to be low molecular weight peptides (<0.5 kDa), with ~90% <1 kDa.[147] Higher abundance of smaller peptides have been described in the literature to elicit important functional activities.[13,15,85] Membrane filtration is a standard approach that can be used to enrich smaller peptides in the aqueous hydrolysate fraction. Generally, both the emulsion and protein hydrolysate fractions appear to have a similar peptide molecular weight profile based on gel permeation chromatography.

Table 2.3: *Peptide molecular weight ranges of salmon emulsion and hydrolysate fractions generated from fermented Atlantic salmon*

MW range (Da)	**Emulsion**	**Hydrolysate**
>10000	1.6 ± 1	1 ± 0.2
5000-10000	1 ± 0.3	1 ± 0.2
5000-3000	2 ± 1	2 ± 0.3
3000-1000	20 ± 1	20.3 ± 2
1000-500	23 ± 1	27.7 ± 2
<500	52 ± 0.1*	49 ± 1

*indicates a statistical significance of $p<0.05$ with paired t-test

While GPC analysis provided a similar mass profile between the emulsion and hydrolysate, the approach is limited in its ability to resolve peptides of similar mass. For this reason, we adopted an MS-based strategy to analyze the molecular weight profile of peptides in the emulsion and protein hydrolysate fractions. Therefore, the mass ranges of precursor ions from LC-MS/MS data are used to compare the peptide molecular weight of hydrolysate and emulsion fractions. Figure 2.2 indicates the mass range of precursor ions. However, it is noted that the LC-MS/MS detection is also subject to biases and has limitations towards the ionization and detection of smaller (<600 Da) and higher (>4000 Da) molecular weight peptides. The hydrolysate and emulsion fractions have comparable peptide molecular weight abundances in the <2500 Da range. In contrast to the emulsion fraction, the hydrolysate fraction has only a few peptides detected above 2500 Da. However, the molecular weight profile is generally similar between the hydrolysate and emulsion fractions.

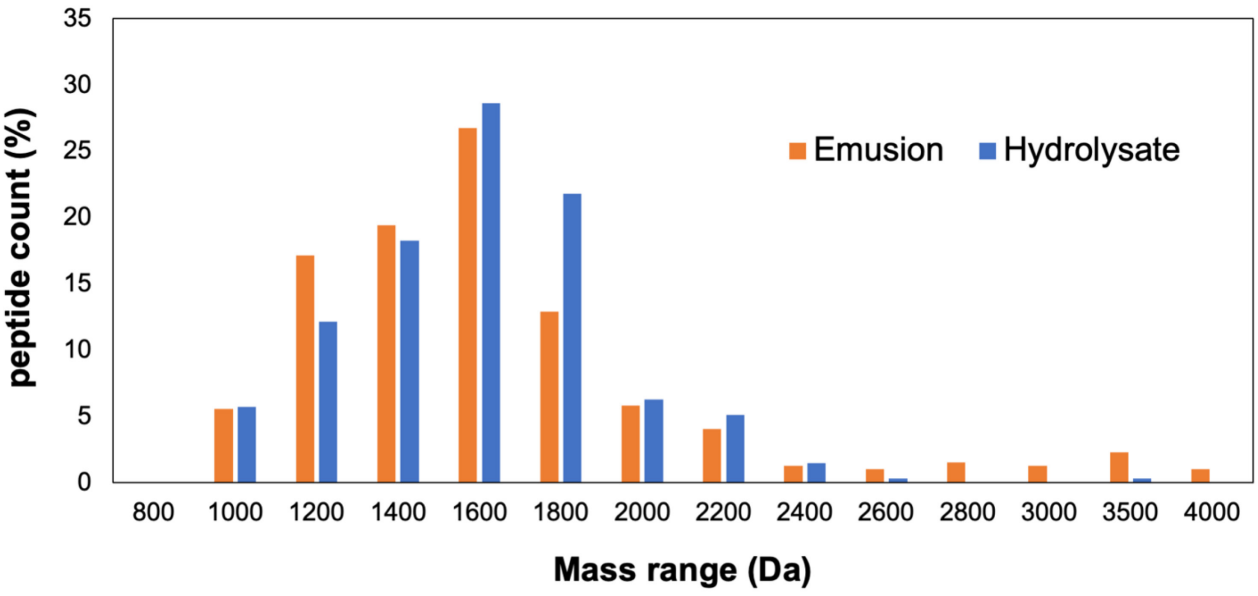

Figure 2.2: *Abundance of precursor ions in various mass ranges in Atlantic salmon emulsion and hydrolysate fractions as determined through LC-MS.*

The total ion chromatogram of the hydrolysate and emulsion fraction on a reversed-phase separation is depicted in Figure 2.3, A. The hydrolysate chromatogram is observed to be temporally separated from the emulsion peptides, which have features with longer retention times. The higher retention of emulsion fraction features can be considered to be as a result of higher hydrophobicities of the constituent peptides, eluting later compared to the hydrolysate fraction.

The peptide identifications were carried out for native peptides in these fractions (without additional tryptic digestion). Higher GRAVY scores are associated with more hydrophobic peptides as the score for a given peptide/protein is calculated by summing the hydropathy values of all constituent amino acids in the sequence divided by the total number of residues in the sequence. Each amino acid is assigned a hydropathy value based on its partitioning between two immiscible liquid phases. Figure 2.3 B indicates the abundance of peptides in various GRAVY

score ranges. It is observed from the Figure 2.3 B that the emulsion fraction has more peptides with higher GRAVY score ranges (0-2), indicating higher hydrophobicity. Conversely, the hydrolysate fraction has higher abundance of hydrophilic peptides with GRAVY scores between -2 and 0. The average GRAVY score of the emulsion fraction was slightly higher at -0.58, compared to -0.70 calculated from the hydrolysate fraction. The average isoelectric points are similar for the peptides identified from emulsion (5.27) and hydrolysate fraction (5.31).

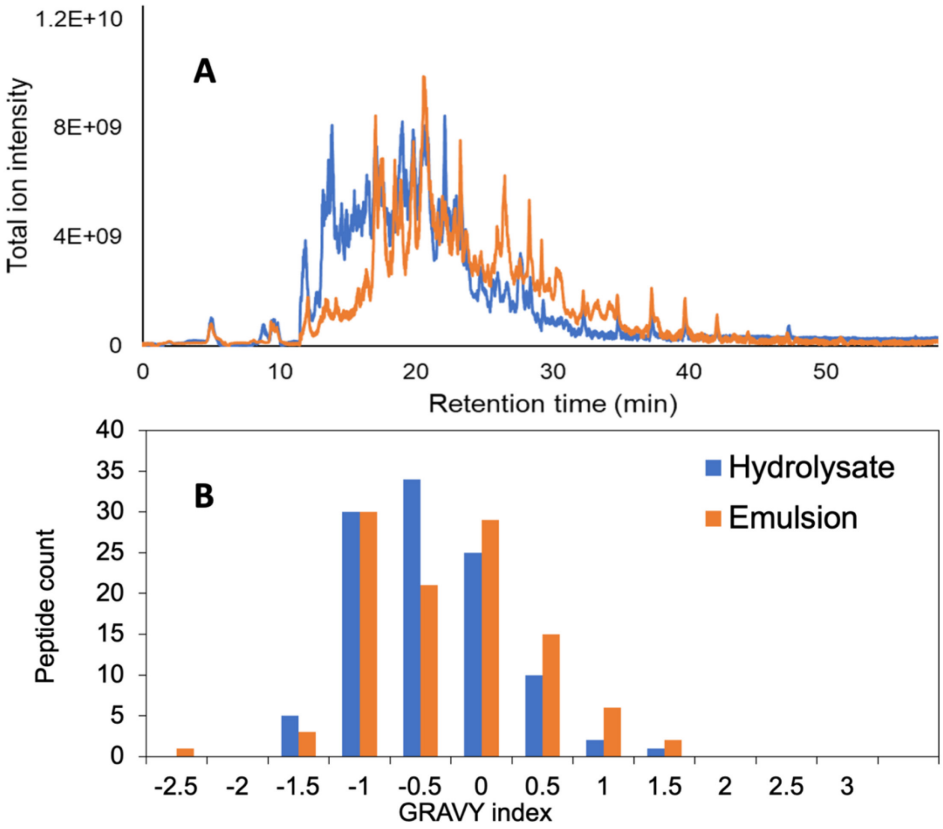

Figure 2.3: *(A) Total ion chromatograms of emulsion and hydrolysate (SH) evaluated on a C-18 columns interfaced with a tandem mass spectrometer (Q Exactive HF-X (Thermo Fisher Scientific, San Jose, CA)). (B) GRAVY score ranges of peptides identified from the emulsion and hydrolysate fraction.*

Proteomic profiling of the two fractions were carried out following the sample preparation steps with SDS extraction (emulsion fraction only), transmembrane electrophoresis (emulsion fraction only), tryptic digestion (both emulsion and hydrolysate fractions) and desalting (both emulsion and hydrolysate fractions). Compared to the hydrolysate fraction, the emulsion required additional sample preparation steps for proteomic analysis on account of the peptides being complexed with the lipids present in the fraction. As seen in the Venn diagrams of Figure 2.4 (A and B), comparison of hydrolysate and emulsion fraction yields low overlap in both identified proteins (19.8%) and peptides (7.3%) lists generated following tryptic digestion. It is acknowledged that differences in sample preparation could also have played a role in the variations observed in the profile of identified tryptic peptides (Figure 2.4, B) in addition to the inherent differences between the emulsion and hydrolysate fractions. Most abundant proteins as determined by spectral counts are listed in the appendix (Table A1 and A2). The peptide lists generated from native peptides in both fractions, where sample preparation prior to analysis was carried out without the tryptic digestion, resulted in significantly shorter lists (Figure 2.4, C) compared to peptide sequencing after trypsin digestion (Figure 2.4, B). It is unclear the reason behind low peptide identifications in both the emulsion and hydrolysate fraction. It is expected that non-specific cleavage offers too many possibilities for spectral matching and confident identification. Additionally, lower ionization efficiency of peptides compared to tryptic peptides could also be a contributing factor. But, similar to other comparisons (Figure 2.4, A, B), a low overlap (~21.6%) was observed in the comparison of inherent peptide sequences identified from the emulsion and hydrolysate fraction (Figure 2.4, C). Even though the hydrolysate and emulsion fractions are subjected to the same processing approach and originate from the same source, the partitioning

during centrifugation generates unique profiles in each fraction as evident from the distinct protein/peptide profiles (Figure 2.4).

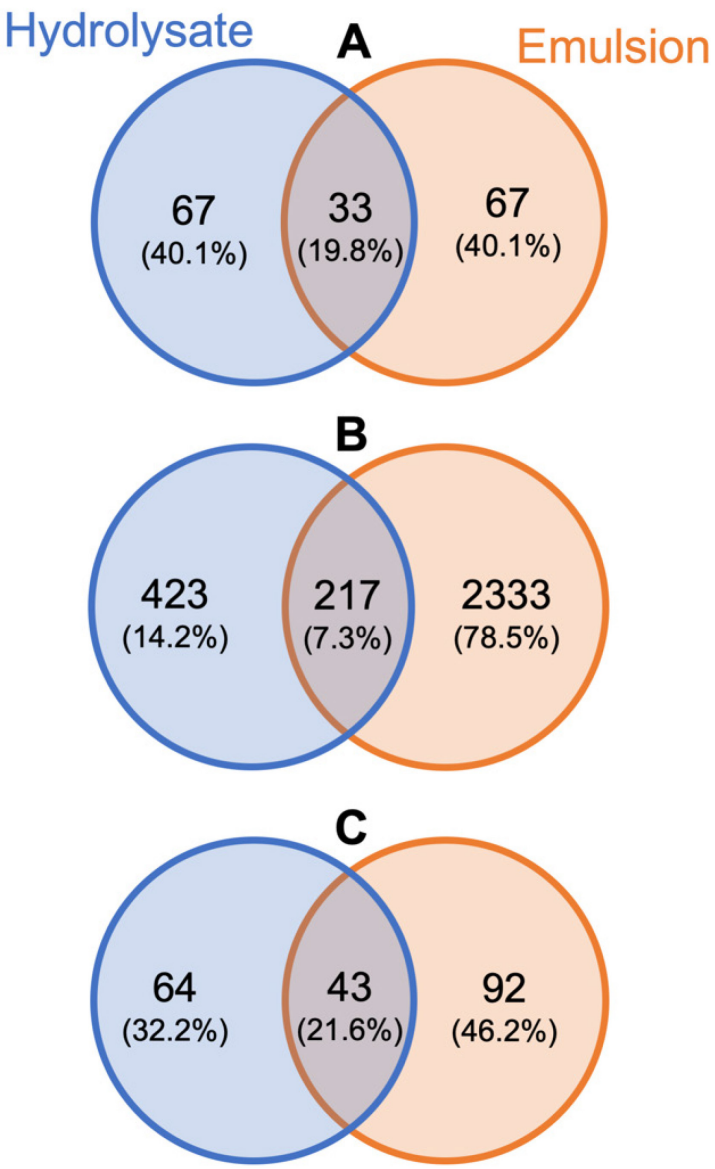

Figure 2.4: *Venn diagrams comparing (A) most abundant (based on total spectrum counts) proteins identified after tryptic digestion; (B) identified peptides following tryptic digestion; (C) peptides identified from hydrolysate and emulsion fraction without tryptic digestion.*

2.3.5. Amino acid composition

The amino acid composition of hydrolysate and emulsion fraction is presented in Table 2.4. Essential amino acid content of the hydrolysate fraction is 39.82%, which is relatively lower than values reported in other marine peptide products reported in the literature.[118,146] These lower levels could be as a result of longer processing durations associated with the microbial fermentation approach compared to enzymatic hydrolysis. Belhaj *et al*. (2013) reported that a phospholipopeptidic complex from Atlantic Salmon was rich in Gly > Glu > Asp > Ala.[93] Similarly, it was observed that the emulsion fraction and hydrolysate fraction from Atlantic Salmon both had higher abundances of Glx (Glu+Gln) > Gly > Asx (Asp+Asn) > Ala. However, compared to the aqueous hydrolysate, the emulsion fraction showed higher abundance of hydrophobic/aromatic amino acids, Phe (60% more) and Tyr (2-fold higher). These trends (higher aromatic amino acid residues in the emulsion fraction) were also observed in the emulsion and aqueous fractions derived from Rainbow trout and Cobia (unpublished results). The emulsion fraction was also found to have 2-fold higher arginine abundance whereas no differences were observed for lysine and histidine. Based on the amino acid composition and peptide profiles (described in previous sections), it can be inferred that the hydrolyzed peptides separated into the emulsion fraction as a result of higher hydrophobicity arising from their constituent amino acids, which enabled the formation of the emulsion fraction. Hatab *et al*., (2017) and Trang and Pasuwan (2018) have inferred that hydrophobic peptides containing cationic amino acid residues can penetrate the bacterial cell to induce antimicrobial activity.[148,149] Therefore, unique features associated with the emulsion fraction peptides have to be further evaluated for other bioactivities such as antimicrobial activity.

Table 2.4: *Amino acid composition of the emulsion and hydrolysate fractions (dried and defatted) generated from fermented Atlantic salmon by-products.*

Amino acids	AA content (µg/mg of sample)	
	Hydrolysate	**Emulsion**
Asx (Asp+Asn)	39 ± 9	41 ± 2
Glx (Glu+Gln)	56 ± 11	55 ± 3
Arg	5 ± 1	11.1 ± 0.2*
Lys	29 ± 4	30 ± 1.3
His	11 ± 2	11.2 ± 0.2
Ser	11 ± 2	13 ± 1
Thr	13 ± 2	15 ± 1
Met	12 ± 2	14 ± 0.2
Gly	52 ± 9	42 ± 2
Ala	38 ± 11	32 ± 4
Val	20 ± 4	22 ± 1
Ile	15 ± 3	19 ± 1
Leu	25 ± 5	30 ± 1
Phe	9 ± 0.7	15 ± 0.3**
Tyr	5 ± 1	10 ± 0.4*
Pro	31 ± 7	26 ± 2

*&** indicates a statistical significance of $p<0.05$ and $p<0.01$ respectively, based on paired t-test

2.3.6. Antioxidant activities

Transition metals generate reactive oxygen species, resulting in lipid peroxidation and DNA damage. Therefore, the chelation of transition metal ions by antioxidant or antioxidative peptides would result in reduction in ROS. The hydrolysate fraction had Fe(II) chelation capacity of 28 ± 8 μM EDTA equivalent. The emulsion was observed to have a 4-fold higher metal chelation capacity (98 ± 1 μM EDTA equivalent), in comparison with the hydrolysate fraction (Figure 2.5, A). The emulsion demonstrated metal chelation capacities comparable to hydrolysates/peptides with high metal chelation capacities described in the literature.[8,27,150] High abundance of aromatic and basic amino acid residues (as in the case of the salmon emulsion fraction) have been observed to increase ferrous chelation capacity in peptides by O'Loughlin *et al*., (2015).[150] However, defatting resulted in a 5-fold decrease in activity. Metal chelating component of the emulsion is disrupted or eliminated during the process of diethyl ether lipid extraction. It is also noted that the defatted peptide suspension had very low solubility which potentially lead to lower bioactivity. The ferric reducing capacity of smaller peptides are reported to be higher in the literature.[84,151] Both the hydrolysate and emulsion (defatted) fractions demonstrated comparable FRAP and act as electron donors to reduce ferric ions as shown in Figure 2.5, B. Emulsion fraction was unsuitable to be used for the assay without defatting.

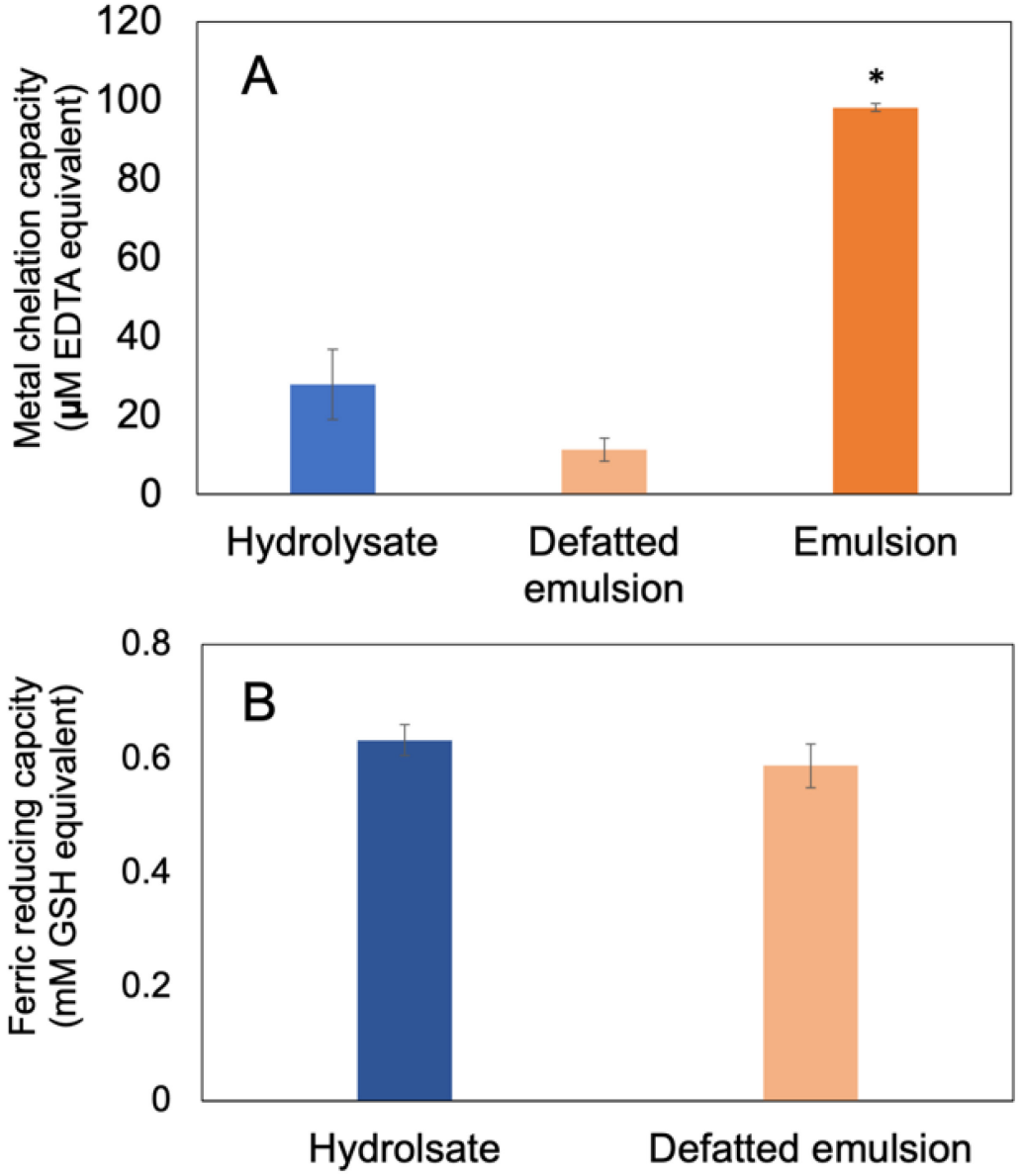

Figure 2.5: *Antioxidant capacity of emulsion and hydrolysate fraction. (A) metal chelation capacity expressed as µM equivalent of EDTA; (B) ferric reducing capacity expressed as mM equivalent of GSH.*

* indicates significant difference of $p < 0.05$ based on multiple comparison of means (Tukeys test) using one-way ANOVA.

2.4. Conclusion

The present study attempted to characterize the fractions generated from microbial bioprocessing of Atlantic Salmon by-products. The protein hydrolysate fraction has potential for several applications in developing functional food because of its high solubility and functional properties. Molecular weight profiles of the hydrolysate and emulsion fractions derived in the present study indicated major contribution of peptides below 1 kDa (~75%) in both fractions. This study provides a better understanding of the underlying peptide behavior that leads to the formation of the emulsion fraction i.e., higher hydrophobicity. While originating from the same source, the hydrolysate and emulsion fractions had distinct peptide profiles. Owing to its hydrophobicity, a potential application of the emulsion fraction will be towards incorporation into food matrices to improve food shelf life and reduce spoilage. The hydrolysate fraction can be further refined to generate peptide products with higher bioactivity with potential to be incorporated.

CHAPTER 3

Evaluation of storage stability of aqueous protein hydrolysate recovered from lactic acid fermentation of Atlantic Salmon by-products

Abstract

Biochemical parameters were studied during storage of salmon protein hydrolysate generated via lactic acid bacterial autolysis. The hydrolysate stored at two different conditions (room temperature and at 4 °C) were stored over a period of 12 weeks. Degradation of larger MW and hydrophobic components were observed in the protein hydrolysate after 8 weeks of storage at room temperature via LC-MS/MS. Similarly, free amino nitrogen content was observed to increase significantly in the hydrolysate after 8 weeks at room temperature (21.3%) and 12 weeks (18.4 %) at 4 °C. However, protein quality assessed via crude/soluble protein content and functionality tested using ferric reducing capacity showed no changes (~0.6 mM glutathione equivalent) over the period of 12 weeks in both storage conditions. The samples over the entire period of storage did not have quantifiable levels of lipid oxidation products. Therefore, the salmon protein hydrolysate is amenable for periods of storage, retaining product quality, especially from the perspective of further processing, transport and storage.

3.1. Introduction

The liquefaction and stabilization of minced whole fish or fish offal has traditionally been performed through the addition of acid. The low pH creates an ideal environment for autolysis by endogenous enzymes, inducing liquefaction and resulting in a mixture of proteins, short peptides, and free amino acids, among other nutritional components such as fatty acids and minerals. A pH below 4.5 is suggested to prevent the growth of microbes, while retaining the pH above 3.5 eliminates the need to neutralize the resulting material before further use.[6] Microbial fermentation is especially sought after in this field as a sustainable approach to generate organic acids which can establish the low pH environment needed to form desirable by-products (such as deproteinized whey). It is essential to understand the stability and shelf life of the fish protein hydrolysates for successful development of bioproducts such as a fortified food/drinks,[117] animal feed or pet food.[36] Storage stability is also relevant for further separation and processing of these materials for the above-mentioned applications.

One of the challenges of using autolysis for the production of salmon protein hydrolysate is the variability of the end product. Processing parameters such as pH, organic acid type/content, and storage conditions, can influence the final composition, nutritional quality, physicochemical properties and functionality of the fish protein/peptide bioproducts.[120] While storage stability of bovine (milk) protein hydrolysate,[120,152] plant proteins[153] and enzymatically generated fish protein hydrolysate[117,118] have been characterized in the literature (reviewed by Rao *et al*., (2016)),[120] the storage stability of fish protein hydrolysate generated by microbial fermentation remained uncharacterized.

The objective of this study was to perform a systematic evaluation of the storage stability of salmon protein hydrolysates, SPH (described in Chapter 2). Evaluation of the impact of storage

at various temperatures and over a range of durations was performed with respect to chemical composition, physiochemical properties and antioxidant activity of SPH. This work contributes to the understanding of the role of storage conditions in influencing feasibility/pitfalls for downstream processing of protein hydrolysate and product shelf life in fish peptide bioproduct development for high-value utilization of salmon by-products.

3.2. Materials and Methods

3.2.1. Assessment of storage stability

The SPH was aliquoted to multiple sealed microcentrifuge tubes and stored at room temperature (~20 °C; RT) and at 4 °C. The sample tubes were withdrawn at different intervals over the course of 12 weeks, after which they were snap frozen in liquid nitrogen and lyophilized prior to further analysis of physicochemical properties.

3.2.2. Protein content

Crude protein content was estimated from total nitrogen content of dried samples which was determined using an elemental analyzer (2400 CHNS/O Series II System, Perkin Elmer, USA). The crude protein content was calculated using the formula as described in equation 2.1.

Soluble protein content in the lyophilized samples was evaluated using modified Lowry assay following their resuspension in phosphate buffer (1 mg/mL) with BSA as a standard.

3.2.3. Free amino nitrogen content

Free amino nitrogen (FAN) content was determined using the method reported by Nielsen *et al*.[62,154] Sample (1 mg/mL sample, 33 µL) was added to 250 µL of o-phthalaldehyde reagent and the absorbance was measured at 340 nm (Tecan M1000 microplate reader, Männedorf, Switzerland). FAN content expressed as serine equivalent.

3.2.4. Evaluation of lipid peroxides

Lipid peroxidation products in stored SPH samples were evaluated using Diphenyl-1-pyrenylphosphine (DPPP, Molecular Probes^{TM}, OR, U.S.A.) as a fluorescent probe. 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 (Tecan M1000 microplate reader, Männedorf, Switzerland).[155] DPPP solution (50 µL of 50 µg/mL dissolved in dimethyl sulfoxide and diluted using 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. Additionally, thiobarbituric acid reactive substance (TBARS) assay was also carried out to evaluate the presence of lipid peroxides in the stored SPH samples.[131]

3.2.5. LC-MS analysis

Purified SPH samples were dried and resuspended in 0.1% FA in 5% acetonitrile. Sample were injected (10 µL) to self-packed spray tips (New Objective, Woburn, MA) with Poros R2 beads (ThermoFisher Scientific) using a dual capillary LC system (Agilent technologies) interfaced to an LTQ linear ion trap mass spectrometer (ThermoFisher Scientific, San Jose, CA).[142] The LTQ operated in data-dependent mode (MS followed by MS/MS of top three peaks) with 30 s dynamic exclusion. The TIC was visualized using MZmine and Peaks Studio ((Bioinformatics Solutions Inc., ON).

3.2.6. Ferric reducing antioxidant potential

The ferric reducing antioxidant potential (FRAP) of the hydrolysates was carried out based on a method by Oyaizu (1986) with modifications.[62,144] Equal volumes (100 µL) of sample (4 mg/mL) and potassium ferricyanide (1% w/v, Sigma-Aldrich, ON, Canada) were mixed and incubated at 50 ºC for 20 min. 100 µL of TCA (10% w/v) was added and the mixture was 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 (Tecan M1000 microplate reader). The ferric reducing antioxidant potential was expressed as mM GSH equivalent based on a GSH standard curve.

3.2.6. Statistical analysis

All colorimetric assays were carried out in triplicate with results expressed as mean ± standard deviation. Fisher multiple comparisons of means was performed with Minitab (USA) to evaluate significant difference ($p \leq 0.05$) between samples and control (SPH, snap frozen at 0 days of storage).

3.3. Results and Discussion

SPH is a clear yellowish solution immediately after collection of the fresh product following centrifugation. Visual inspection of the samples over the course of storage showed the progressive development of a brownish color. This was most prominent when stored at RT. Figure 3.1 depicts the difference in color development depending on the storage condition. Samples stored at RT exhibiting a noticeable brown color at 8 weeks. The samples stored in the fridge gave the appearance of a slight brown color at 12 weeks. A previous study from our group demonstrated that ~0.2 mM lactose (used in fermentation) remains after fermentation, which can potentially lead to a Maillard reaction (non-enzymatic browning).[120] However, the acidic environment of the SPH should reduce the formation of non-enzymatic glycation products.[156] Additionally, during fermentation and storage, the bacterial decarboxylation of amino acids or transamination of aldehydes and ketones can lead to formation of active biogenic amines which are nitrogenous organic bases of low molecular weight, polar or semi-polar compounds.[157] Formation of these

biogenic amines can also be potentially responsible for the appearance of brown color in the samples stored at RT after 4 weeks. It is noteworthy that the appearance of the brownish color did not correspond with turbidity in the SPH; all solutions remained clear with no cloudiness at RT or at 4 °C with up to 12 weeks storage. This therefore is an indication that microbial growth did not occur. Growth is predicted to be inhibited by the low pH and potential antimicrobial compounds generated by lactic acid bacteria during bioprocessing. The lack of turbidity is also an indication that protein aggregation did not occur. This would be a major concern for samples containing high protein/peptide concentrations.[120]

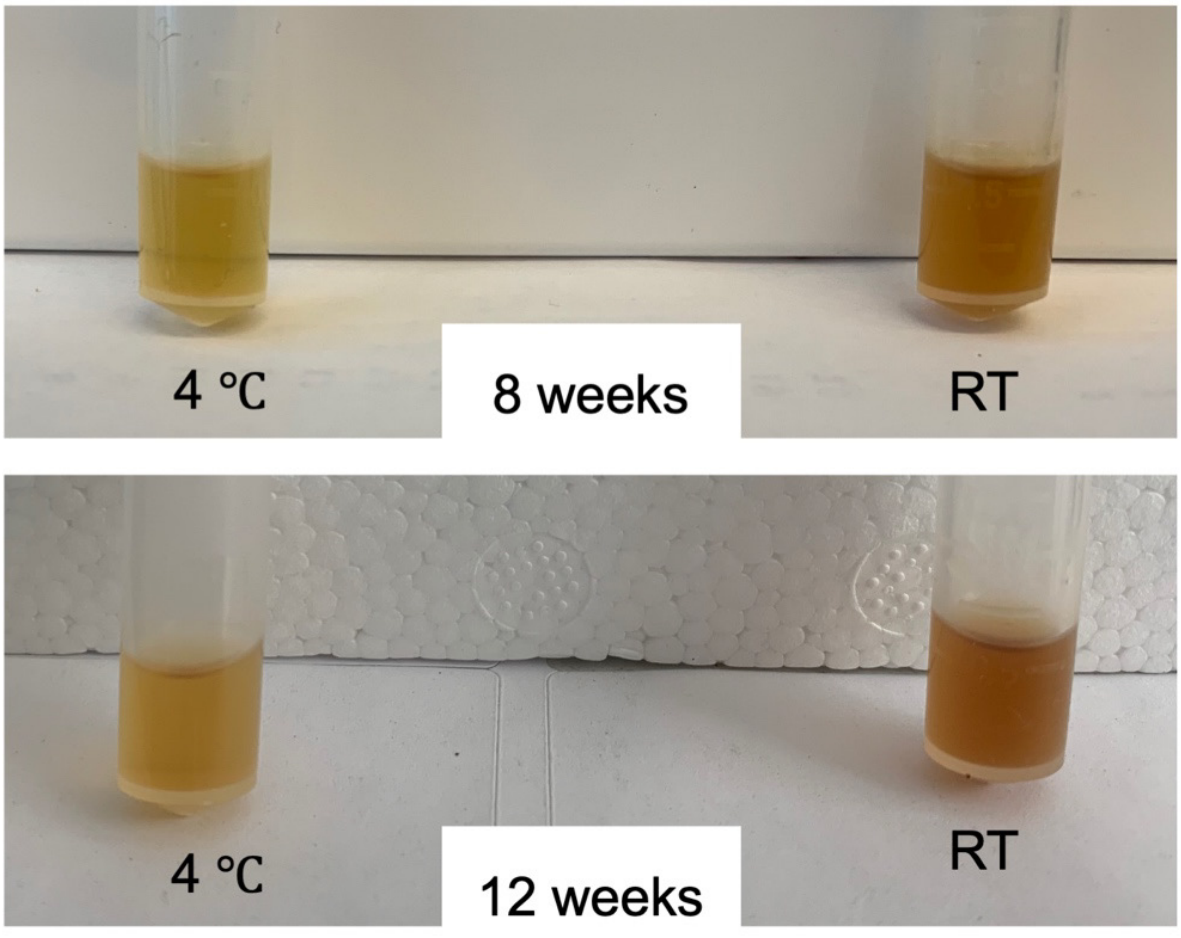

Figure 3.1: *Photograph of SPH extract stored at 4 °C and at room temperature (RT).*

Oxidation of myoglobin and melanin pigments is also reported to result in darkening of the hydrolysate color.[118] Additionally, lipid peroxidation could lead to development of this darker color. Unnikrishnan *et al*., (2020) quantified the malonaldehyde (lipid peroxidation product) content to range from 0.9-3.6 mg/kg in yellowfin tuna peptides during six-month storage in ambient conditions and 0.9-2.2 mg/kg in fridge storage.[118] In contrast, lipid peroxide estimation carried out fluorometrically using DPPP and colorimetric detection using TBARS assay did not quantify detectable levels of products of lipid peroxidation in any of the SPH samples (stored at RT or 4 °C). Therefore, the development of brown coloration was not as a result of lipid peroxides. The lack of lipid peroxidation products is an indication of SPH product quality with longer shelf life and reduced deterioration of nutritional and sensory qualities.

3.3.1. Protein content

Biogenic and volatile amines that are formed as a result of free amino acid metabolism during storage.[158] These metabolic products can result in lowering of the protein content and N content in SPH.[158] However, based on the crude protein content calculated from total nitrogen content, statistically significant differences were not observed ($p > 0.05$) in the samples stored at 4 °C or at room temperature. The samples varied between ~53% to 56% crude protein content on a dry matter basis. Production of volatile amine products can result in a decrease in total nitrogen content, and thereby the crude protein content, which was not the case up until 12 weeks of storage at room temperature. Similarly, the soluble protein content of stored SPH samples (4 °C and RT) was not observed to have significant changes ($p>0.05$). Therefore, total protein content remained unaltered in SPH in case of room temperature and fridge storage. Similarly, Thiansilakul *et al*., (2007) observed no substantial differences in protein solubility for fish protein hydrolysates kept at 4 °C and 25 °C throughout a 6-week period of storage.[159]

3.3.2. Properties of stored SPH samples

Monitoring the free amino nitrogen (FAN) content is an indication of further degradation of peptides. As partially hydrolyzed proteins are further hydrolyzed to smaller peptides or free amino acids, the FAN content is expected to increase.[8] Figure 3.2 depicts the change in FAN contents in the stored SPH samples. The increase in FAN content at 8 weeks for room temperature storage (305 to 370 μM serine equivalent, 21.3% increase) and 12 weeks for 4 °C storage (361 μM serine equivalent, 18.3% increase) is indication of hydrolysis of peptides.

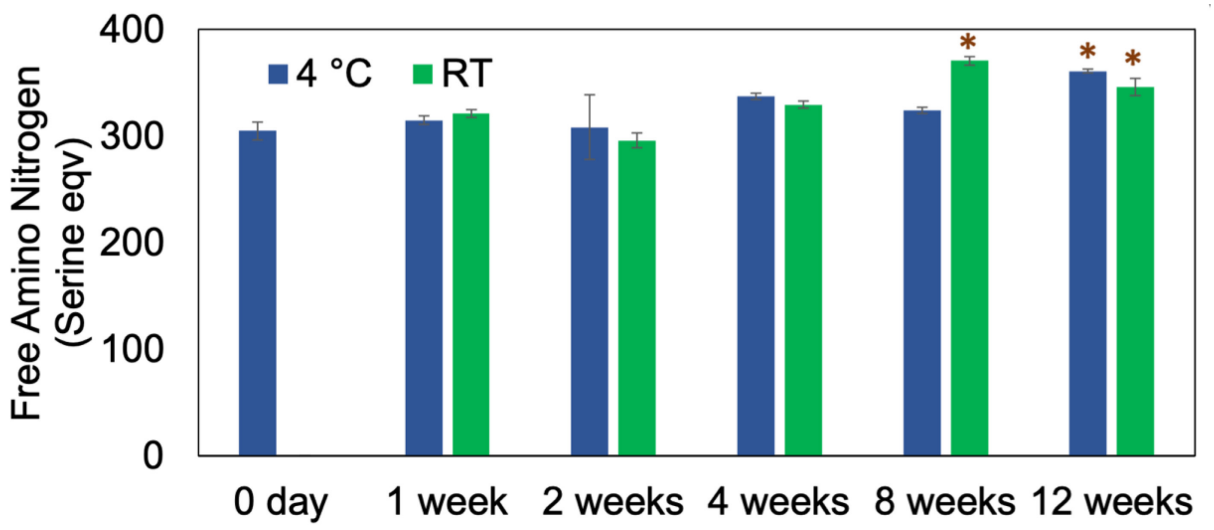

Figure 3.2: *FAN content of SPH stored at 4 °C (F) and RT over the course of 12 weeks.*

* indicates significant difference at 95% confidence with Fisher multiple comparison of means using one way analysis of variance, time 0 was used as the control

Ferric reducing antioxidant potential (FRAP) determines the ability of the samples to function as an electron donor to reduce ferric ions. An increase in the degree of hydrolysis of peptides has been described to result in reduced FRAP activity.[8,27,92] However, FRAP activity did not decrease ($p > 0.05$) in the SPH samples stored for 12 weeks at either temperature (~0.6 mM

GSH equivalent). Similarly, Thiansilakul *et al*. (2007) observed stable ferric reducing capacity in marine peptides generated from round scad during a period of 6 weeks (both at 25 and 4 °C).[159] However, unlike the present study, Thiansilakul *et al*. (2007) performed the storage stability evaluation on dried protein hydrolysate.[122] Additionally, the presence of moisture has been regarded as a major detrimental factor in reducing the stability of protein hydrolysates.[117,118] Therefore, this is a further indication of the stability of the liquid salmon protein hydrolysate in the present study.

3.3.3. LC-MS profile of SPH samples

Previous studies have demonstrated that proteolytic activity and microbial load (colony forming unit count) are reduced to negligible levels after a week of bioprocessing in SPH samples.[8,92] Therefore, it was expected that, even without heat-induced enzyme inactivation, SPH would remain stable for a month without significant changes. As expected, further hydrolysis of samples was not observed.

Following 8 weeks of storage at room temperature, several features were observed to have been diminished in the LC-MS profile (Figure 3.3, A). This was especially pronounced for peaks with retention time >40 min, representing the more hydrophobic peptides, which tend to be larger as well. It was observed that these MS features becomes negligible at the 8th week of storage at RT (Figure 3.3, A). In contrast, SPH storage at 4 °C was observed to have consistent features (Figure 3.3, B). Lower peak intensities for SPH stored at 4 °C were only observed for sample stored for 12 weeks (Figure 3.3, B). Similarly, Grave *et al* (2016) demonstrated that temperature of storage rather than pH played a significant role in determining the stability of a plant-derived peptide.[153]

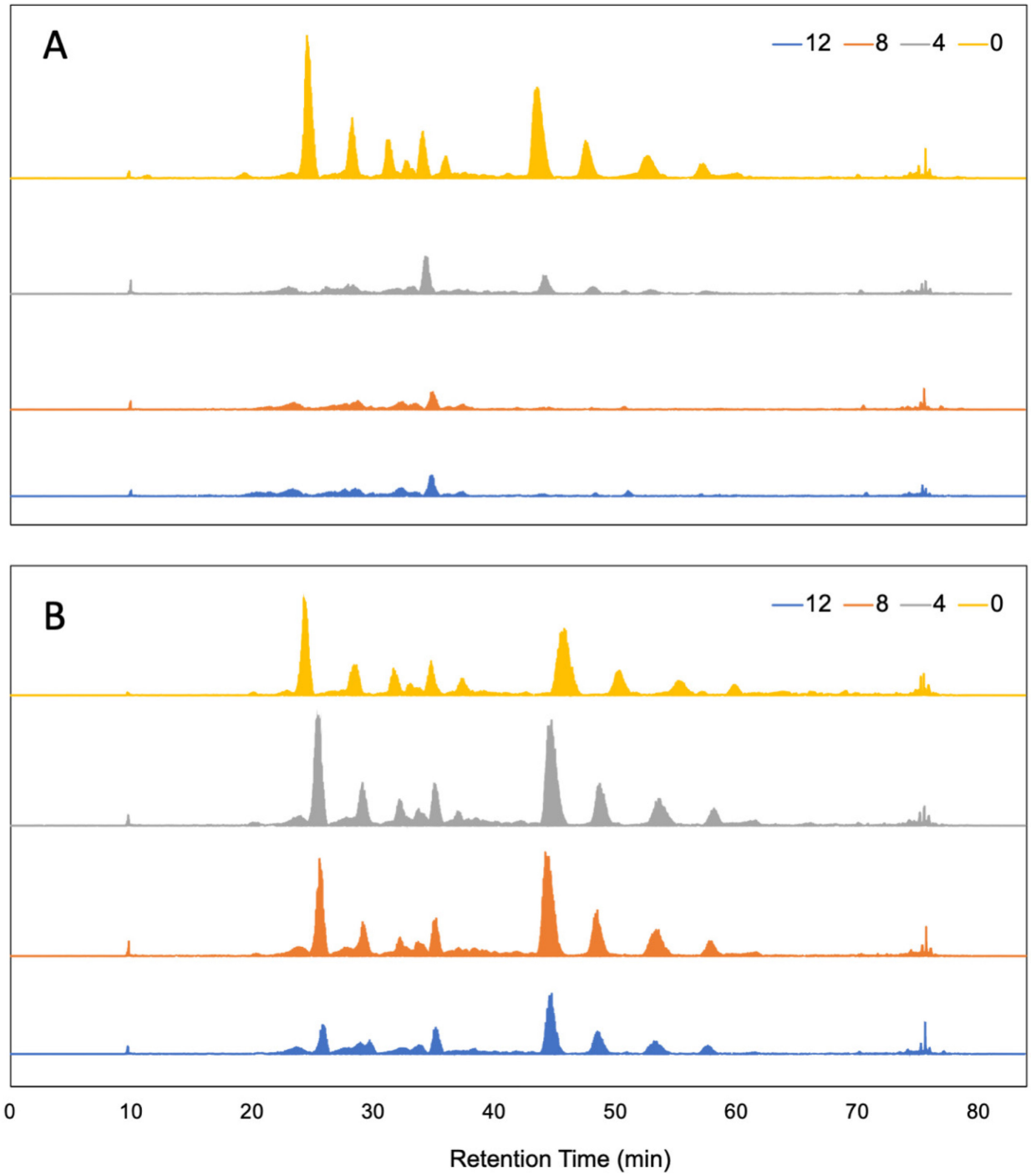

Figure 3.3: *Total Ion Current (TIC) trace of base peak intensity with the corresponding retention time derived from the LC-MS analysis of SPH samples stored over a period of 12 weeks (A) at RT; and (B) at 4 °C. Figure legend: 12, 8, 4, 0 indicates the storage duration of sample in weeks.*

MS features between *m/z* 500-600 and *m/z* ~900 were observed to have been degraded over the course of the storage at RT stored for 8 and 12 weeks (Figure 3.4). Additionally, major features between *m/z* 500-600 with retention times 20-40 min also demonstrated reduced signals in R4, R8 and R12 (Figure 3.4). In the case of storage at 4 °C over a 12-week period, MS features remain consistent for the hydrolysate over the storage period, with a slight decrease in peak intensity seen at the end of the 12-week storage (Figure 3.4). Conversely, a progressive increase is observed in the abundance of initially eluting (unretained on column) peaks were observed with storage at RT. These peaks are expected to be highly hydrophilic degradation products generated during the storage period (more so at RT). In the case of storage at 4 °C, these early retention time LC-MS features were observed to be less prominent, and can only be observed at 12 weeks of storage. The larger peptides in the protein hydrolysate are predicted to be degraded during RT storage resulting in smaller hydrophilic molecules.

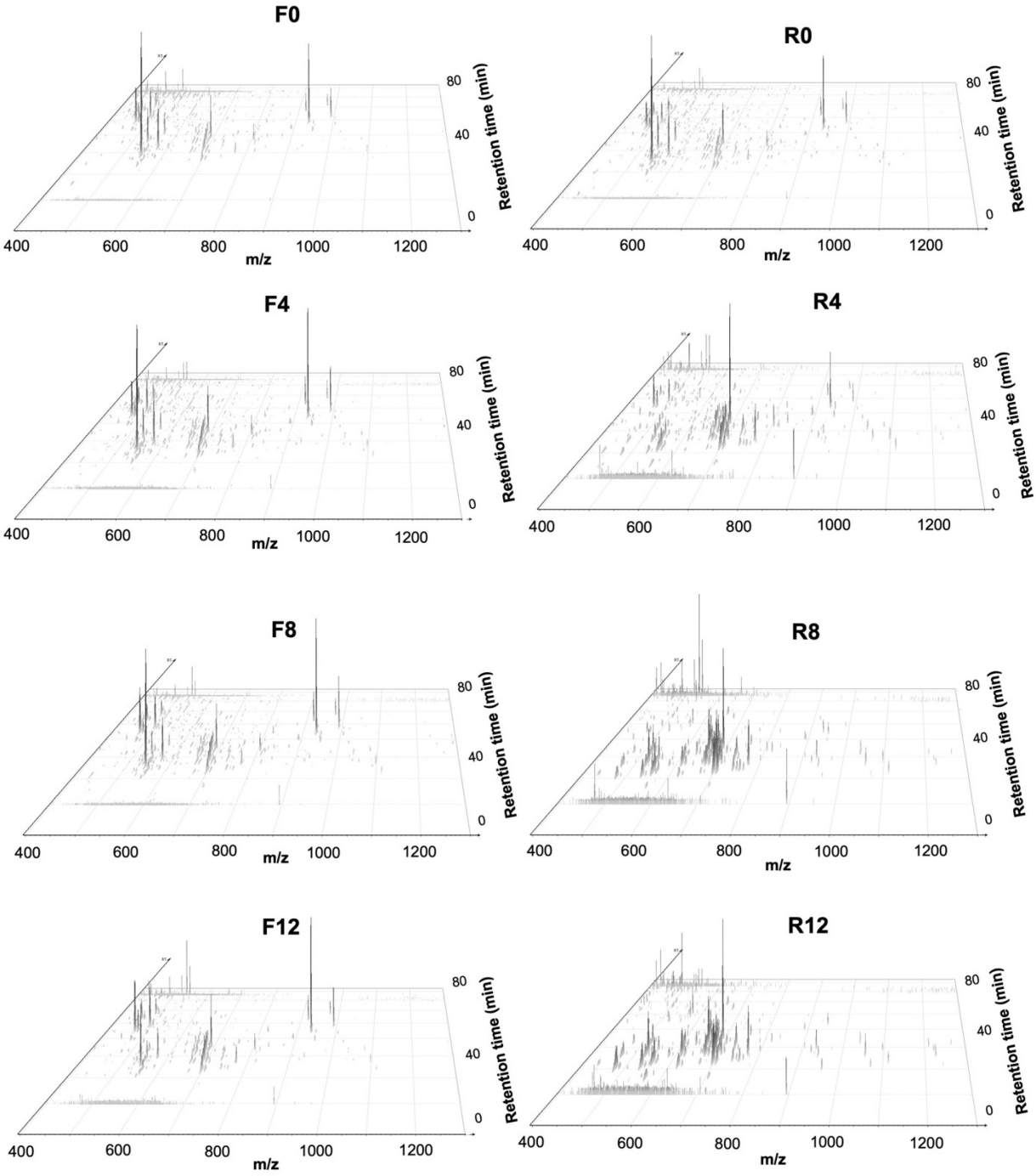

Figure 3.4: *3D intensity view for visual inspection of the LC/MS data of SPH samples stored at room temperature (R) and 4 °C (F). The numbers 0, 4, 8 and 12 associated with R and F indicate the storage period in weeks.*

3.4. Conclusion

Examination of the SPH degradation over time demonstrated that room temperature storage can reduce the peptide features present, even though functionality and physicochemical characteristics remained mostly intact. Crude protein content of ~55% for the SPH remained intact for a period of 12 weeks. Peptide stability evaluated by mass spectrometric analysis revealed a stability for up to a month under ambient conditions whereas storage in the fridge improved stability up to 12 weeks (and possibly more). Information on storage stability influences product placement and marketability as an ingredient for functional foods or nutraceuticals. Foods incorporated with SPH bioactive peptides will have a potential to serve nutritional and functional potential.

CHAPTER 4

Review of membrane separation models and technologies: Processing complex food-based biomolecular fractions

Abstract

There is growing interest in the food industry to develop approaches for large-scale production of bioactive molecules through continuous downstream processing, especially from sustainable sources. Membrane-based separation technologies have the potential to reduce production costs while incorporating versatile multi-product processing capabilities. This review describes advances in membrane technologies that may facilitate versatile and effective isolation of bioactive compounds. The benefits and drawbacks of pressure-driven membrane cascades, functionalized membranes and electromembrane separation technologies are highlighted, in the context of their applications in the food industry. Examples illustrate the separation of functional macromolecules (peptides, proteins, oligo/polysaccharides, plant secondary metabolites) from complex food-based streams. Theoretical and mechanistic models of membrane flux and fouling are also summarized. Overcoming existing challenges of these technologies will provide the food industry with several attractive options for bioprocessing operations.

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4.1. Introduction

Food and feed formulations require the bioactive molecules at higher concentration. Since bioactive components are diluted during the formulation process (encapsulation or addition to a complex matrix). Enrichment/separation/purification strategies play a major role towards the development of functional food or nutraceuticals. The ability to generate functional food ingredients from under-utilized natural sources is an attractive, timely and opportune area of research and development, though it still presents challenges to food-ingredient manufacturers because of the complex, heterogenous nature of these materials. Biovalorization processing can be accomplished via: i) large-scale pre-treatment of biological materials, ii) extraction or isolation of compounds of interest, iii) separation of molecular fractions, iv) purification of compounds of interest, and v) incorporation into food/ nutraceutical product formulation.[160] Membrane filtration can be a valuable tool for these processing steps to target the enrichment of specific compounds from food-based matrices,[160,161] with multiple therapeutic and nutraceutical applications.[134]

Membrane filtration is a promising technology for process-scale separation and purification of biomolecules in several diverse industries. The replacement of traditional modes of commercial-scale separation such as chromatography with membrane-based approaches has distinct advantages. Not only are membrane approaches amenable to continuous downstream processing of bio-based products, but they can afford lower operational expenses and safer operation at lower pressures.[162] Pressure-driven membrane separations including ultrafiltration (UF) and nanofiltration (NF) are key non-destructive approaches for processing in several applications such as water treatment, paper/pulp production, fertilizer, petroleum, textile and food industries. The applications discussed in the present review are limited to food processing.

Although membrane technologies offer potentially high separation speed and throughput, the accumulation of solute molecules at the membrane surface, termed fouling, is a major hindrance that has limited their widespread application.[163] Fouling is especially concerning for application of membrane separations in complex food-based feed solutions. To overcome this, the present review highlights theoretical models which form the basis of current and potential innovative developments in membrane-based separations to enhance fractionation/purification of complex matrices with relevance to the food industry. Additionally, current use of membrane-based separations is largely limited to fractionation on the basis of molecular weight, restricting selective separation in downstream processing of complex substrates. The capacity to separate biomolecular fractions from these feedstocks based on their physicochemical properties, such as charge, molecular weight or monomer composition, can aid in the development of bioactives and functional foods. This chapter describes several approaches to improve upon these constraints to enhance selectivity and throughput of membrane processing for applications in food-based systems.

4.2. Membrane filtration *vs.* chromatography

Given their high selectivity and versatility, chromatographic approaches are generally favored at both the analytical and industrial scale for complex separations. Industrial-scale monolithic,[164] counter current[165] and ion exchange[166] chromatography approaches are used extensively in high value biopharmaceutical applications involving separation of biomolecules. However, in addition to high capital and operational costs, traditional chromatographic approaches suffer from low diffusion rates (mass transfer) for macromolecules and ultimately provide low throughput. While offering higher selectivity, chromatographic approaches also tend to require additional steps, either pre- (solvent evaporation, sample filtration) or post-separation (solvent

removal or exchange) which also adds to their drawbacks. Membrane-based separations offer a complementary approach to overcome these limitations. Membrane filtration processes require inherently lower capital costs, they are relatively insensitive to diffusional resistance of macromolecules, and can be easily scaled to high flow rates, affording improved throughput for bulk processes.[167] Contrasting with the batch form inherent to chromatographic approaches, membrane-based processing also affords the potential for continuous operation.[167] While it is recognized that even the most efficient filtration unit cannot provide the selective separations inherent to high efficiency chromatographic columns, enhanced selectivity can be gained by employing multiple membrane separation modules, also known as multistage filtration or membrane cascades. The use of functionalized membranes (eg. charged or permselective) can also enhance membrane selectivity.[168] Another approach to improve the selectivity involves electrodialytic separations using UF membranes, which function under an applied electric field to separate compounds based on their charge. Electrodialysis approaches are gaining in popularity, especially in food applications to separate charged species.[169] These and other membrane-based technologies will be discussed in detail in this review, highlighting their potential for efficient separation and recovery of biomolecules from food or natural products.

4.3. Membrane separation efficacy and fouling: mechanisms and modelling

UF and NF are generally pressure-driven processes by which the desired macromolecular solutes are selectively separated from other impurities through a membrane. Efficiency of these processes is determined by the fraction of material that passes through the membrane, known as permeate flux or flux through the membrane. Various theoretical models have been presented that provide a fundamental understanding of the factors that govern permeate flux across filtration

membranes. These models therefore form the basis for designing improved filter-based separation platforms.

4.3.1. Permeate flux and solvent permeability

The hydraulic permeability coefficient (L_p) is a term used to describe the magnitude of the solvent flux (typically water) through a porous membrane. It can be described mathematically as follows:

$$L_p = \frac{\varepsilon r^2}{8\mu\delta_m} \quad \ldots(4.1)$$

where ε is the membrane void fraction (the ratio of volume occupied by open pores in the membrane), δ_m is the membrane thickness, μ is the solvent viscosity and r is the average radius of the membrane pores, which is generally larger when selecting higher molecular weight molecules. Based on this equation, several filtration parameters can be modified to increase permeate flux, and thereby improve performance. For example, the permeability coefficient is influenced indirectly by temperature as it in turn relates to viscosity. The viscosity of water decreases 3-fold as temperature increases from 4 to 50 °C.[170] As a consequence, higher membrane performance can be observed at elevated temperatures (35 to 40 °C).[171,172] Unfortunately, beyond a threshold (~50°C), higher temperatures are generally unsuitable for biomolecular processing, as most membranes and biomolecules tend to be thermally labile. The viscosity of high concentration protein solutions can be decreased by adding co-solutes such as histidine and trehalose (to disrupt intermolecular interactions), which improved permeate flux across UF membranes.[173] Similarly, thinner membranes (low δ_m), or those with higher pore density (high ε) can improve membrane performance. For instance, the increase in porosity gained from the controlled chemical

degradation of a polystyrene membrane (26 % weight decrease) was shown to result in a 67% increase in flux across a broad pH range.[174]

4.3.2. Solute behavior and gel polarization

Membrane filtration can be employed with a goal of isolating smaller molecular weight species from the feed as they pass through the membrane and are collected in the permeate. Alternatively, filtration also enables the enrichment and collection of desired macromolecules in the retentate. In both cases, as more feed is processed, the flux through a membrane will be impeded by molecules in the feed solution, particularly at high solute concentrations. During a membrane filtration process, as pressure drives solvent through the membrane pores, a fraction of the permeable (low molecular weight) solute will pass through (C_p). Simultaneously solute molecules will accumulate on the surface of the membrane as it is a bottleneck (Figure 4.1).[163] Thus, the solute concentration is highest at the wall (C_w), and the concentration decreases with increasing distance from the surface (wall) of the membrane to the bulk (feed) solution concentration (C_b). The distance from the membrane surface, at which the concentration of the solutes reaches that of the bulk feed (C_b) and is referred to as boundary layer thickness (δ) (Figure 4.1). This change in solute concentration results in the emergence of concentration gradients at the membrane-solvent interface which is termed concentration polarization or gel polarization. Filtrate flux (J_V) is derived from the stagnant film model,[175]

$$J_V = k\ln\left(\frac{C_W - C_P}{C_b - C_P}\right) \quad \ldots(4.2)$$

where k is the solute mass transfer coefficient, which is the ratio of the diffusion coefficient of solute to the boundary layer thickness ($k=D/\delta$). Based on equation (ii), flux across the membrane (J_V) can be improved by enhancing the back-diffusion process, i.e., improving the mass transfer between the boundary layer of solute (on the membrane surface) and bulk solution/feed.[173] Hung

et al. (2016) were able to use this approach with the help of co-solutes to significantly improve flux across UF membranes for protein solutions. Co-solutes such as histidine and imidazole reduce the attractive protein-protein interactions that hold together the boundary layer of protein on the membrane surface thereby improving mass-transfer between the bulk feed and membrane wall.[173]

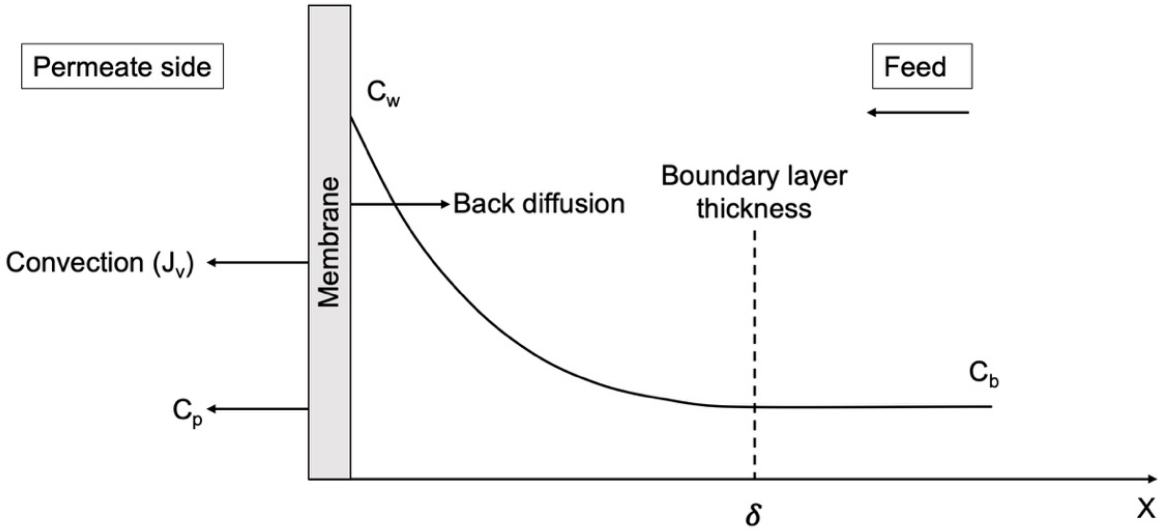

Figure 4.1: *Schematic diagram of solute transport across a membrane boundary of δ via convection and diffusion (Adapted with permission from Eztel (2009) Copyright {2009} [Wiley and sons]). The x-axis indicates the distance from the membrane surface and the y-axis indicates concentration of solute*

Similarly, changing feed pH has been shown to suppress intermolecular interactions and improve permeability.[176]

4.3.3. Membrane cascades: Improved selectivity

In addition to solvent permeability and solute behavior, membrane selectivity is a major factor that influences filtration performance. Selectivity of membranes is quantified using sieving

coefficients. The observed sieving coefficient is a fundamental measure of filtration performance and can be defined as:

$$S_o = C_p / C_b \qquad \ldots(4.3)$$

where C_n refers to solute concentration in the permeate (p) or in the bulk feed (b). Selectivity (ψ) between multiple solutes during membrane separation is the ratio of their sieving coefficients:

$$\psi = S_{o1} / S_{o2} \qquad \ldots(4.4)$$

where S_{o1} and S_{o2} are the observed sieving coefficients of the less and more retained biomolecular fractions, respectively (Mehta and Zydney 2005). It is important to note that sieving coefficients are not physical properties of the solutes but rather are process-dependent. Therefore, process parameters can be varied to tailor the selectivity of the membrane.

Gunderson *et al*. (2007) used a combination of membrane modules called diafiltration units to influence flow conditions of the solvent. Diafiltration is the simplest form of membrane cascade (Figure 4.2 a) and involves two stages. Solute concentration in the bulk (C_b) and membrane wall was reduced by dilution of the feed stream, thereby increasing global selectivity (via increased S_o values) and enhancing separation (Figure 4.2).[177] The first stage (Figure 4.2 a (1)), is selectively permeable to low molecular weight solutes, while the second downstream filter (2) is permeable only to the solvent.[177] Diafiltration units can form an important component of a cascade process as a means of removing low-molecular weight impurities while simultaneously facilitating solvent recycling. For instance, a two-stage ceramic ultrafiltration cascade was successfully used to recover (~97%) and purify bromelain from crude pineapple wastes.[178]

In ideal cascades, transmembrane fluxes at each membrane module are distinct as a result of differences in partitioning of the components of the feed solution into permeate and retentate fractions. The number of stages in a membrane cascade relates to enhanced separation efficiency, as does the manner in which the permeate/retentate streams within the cascade are directed (in terms of optimized flow rates, transmembrane pressure).[167,179,180] Figure 4.2 b describes the flow and partitioning of retentate and permeate fractions at various stages of a three-stage membrane cascade. The feed is partitioned initially (Membrane F) and both the retentate and permeate fractions from 'F' are further processed using the W and P membrane modules, respectively (Fig. 2 b). Patil *et al*. (2014) found the ratio of the sieving coefficients (as measured by the concentration solutes in feed and filtrate) in a mixture of solutes to play a major role in influencing yield and purity of solutes.[181] At higher ratios, both yield and purity of solute fractions were found to increase proportionately in a three-stage membrane cascade. In the same study, for a five-stage cascade, the yield and purity became inversely related especially at higher ratios of sieving coefficients i.e. a decrease in yield was observed for fractions with higher purity.[181] Rizki *et al*., (2020) demonstrated that increased temperature during the operation of a 3-stage cascade for separation of oligosaccharide lead to an increase in the product in the permeate flow along with a higher flux, with no change in product purity.[182] Additional study from the same group tested multiple configurations and operating parameters of a three-stage cascade to increase purity of the collected fractions.[183,184] Therefore, cascade system installation and optimization for various solutes is dependent on modelling the sieving coefficients. Sieving coefficient ratios need to be generally low, for optimal separation and satisfactory yield, at higher stages of cascading.

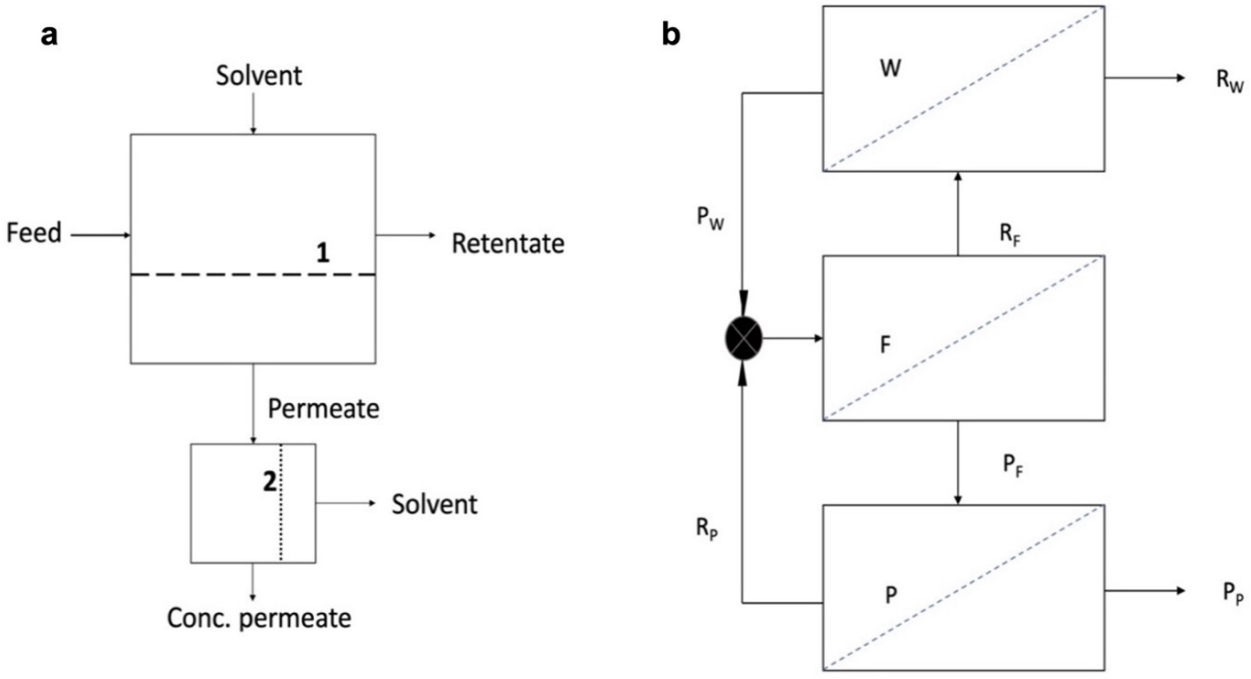

Figure 4.2: *(A) Schematic diagram of solute transport through a diafiltration system that facilitates concentration of high molecular weight solutes and solvent recycling (Modified from Gunderson et al. (2007)). (B) An ideal 3-stage membrane cascade with three modules (W, F, P) and Pi and Ri indicating permeate or retentate flux arising from respective membranes. Adapted with permission from Patil et. al. Copyright [2014] [Elsevier B.V.].*

4.3.4. Membrane fouling: Pressure-limitations

Flux decline is a major factor that has constrained the application of membrane processing, as it necessitates frequent halting for periodic back-flushing to restore flux. Gradual decline of permeate flux is a prevalent phenomenon and occurs primarily as a result of pore blocking and gel layer formation.[163] Figure 4.3 describes the change in flux in different stages as a result of different chemical mechanisms. The rapid drop of flux in the initial phase of the filtration process is

attributed to the membrane pores being blocked by the solute molecules (Figure 4.3 (I)). In phase II, a gradual rate of reduction of permeate flux occurs as a result of formation of the gel layer as the amount of retained solute particles increase on the membrane surface (Figure 4.3, II). The retained layer reaches an equilibrium thickness when particles on the membrane surface layer are in equilibrium with particles in the bulk solution, causing the permeate flux to stabilize and reach a steady state (Figure 4.3, III). In the steady state, the amount of solute material from the feed (bulk) accumulating at the membrane will be equivalent to solute released from the membrane, either as permeate or through back-diffusion of solute in the direction of the feed (bulk).

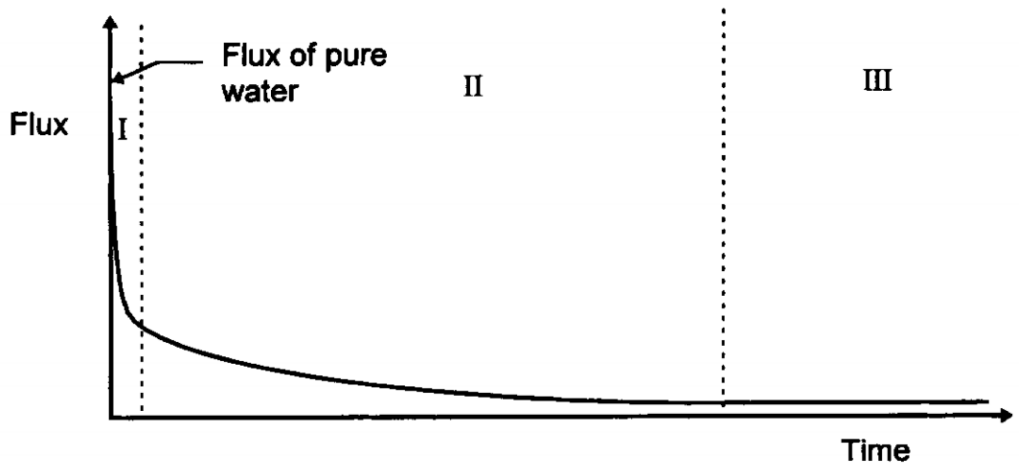

Figure 4.3: *Schematic diagram of different stages of flux decline (Adapted with permission from Song (1998) Copyright [1998] [Elsevier B.V.]).* ***I*** *occurs as a result of solute molecules causing pore blocking,* ***II*** *is a result of solute absorption (cake layer),* ***III*** *is the flux at equilibrium or steady state.*

A critical pressure threshold dictates the formation of a steady state at any given solute concentration. When the applied pressure exceeds a certain critical pressure, the filtration is in

non-equilibrium operation, causing solute to accumulate and expand the thickness of the gel layer, which leads to membrane fouling.[163] There are several models that describe the build-up of solute molecules on membrane surfaces based on the applied pressure.[185,186] The gel polarization model demonstrates flux decline as a function of the mass transport coefficient of the solute while the osmotic pressure model also takes into account variables such as transmembrane pressure.[185] Wijmans *et al*. (1984) compared these two models and clearly showed the flux-pressure profile in UF to be a function of the flux-pressure derivative. According to this model, buildup of solute on the wall of the membrane (C_W) generates an osmotic pressure that counteracts the applied pressure (ΔP). The contributions of fouling and gel polarization and their modelling have been described in detail by Yazdanshenas *et al*. (2010) for clarification of apple juice by an industrial-scale UF module.[186] Identification of flux decline mechanisms is essential for system design and flux enhancement strategies. Additionally, fouling mechanisms vary based on membrane and solute characteristics. Habibi *et al*. (2020) demonstrated that same amount of glutathione and BSA accumulated on the membrane surface generates different levels of flux declines that are dependent on the mechanism of fouling.[187]

As an alternative to dead-end filtration, the crossflow filtration approach places the feed stream flows tangential to the membrane surface and is modelled by a combination of Navier-Stokes and Darcy equations.[188,189] Studies have demonstrated that crossflow UF with low pressure, low substrate load (feed concentration) and low tangential flow velocity demonstrated the higher efficiency for longer term operation.[190,191] In crossflow filtration, spatiotemproral progression of solute deposition (equilibrium and non-equilibrium region) occurs from the feed end to the retentate end of the membrane during processing. The average permeate flux for the entire membrane surface has been modelled by Song, (1998).[163] Crossflow models enable the prediction

of steady state and corresponding flux based on the parameters of design and operation of filtration process.[188,189] However, studies focused on improving the membrane filtration process for macromolecular solutes by the optimization of parameters (transmembrane pressure, particle size, resistance of the gel layer and cross-flow rates) still rely on the basic understanding of membrane filtration.[173,177,192,193] An improved understanding of the chemistry underlying the dynamics of molecules within filtration systems will facilitate fabrication and design of improved filtration systems. Research in this direction is critical for the development of a membrane processing framework for working with feed solutions with multiple solutes and testing the validity of these models.

4.4. Electrically enhanced membrane processing

Modification of membrane surface chemistry can significantly increase flux and reduce fouling. However, despite improvements resulting from surface modifications, pressure-driven membrane systems are still limited by the applied transmembrane pressure. To increase flux without having to increase transmembrane pressure, an electode-assisted filtration (electro-ultrafiltration) approach has been developed based on an electrophoretic process for ion selection in tandem with conventional membrane modules.[194–196] All charged particles migrate in the presence of an electric field, with the anions toward the anode and cations toward the cathode. And the velocity of these charged particles under the influence of an electric potential is determined by their size, viscosity of the medium (feed) and the magnitude of charge and the electric field. Electrophoretic velocity, ν_{ep} is described as

$$\nu_{ep}=\mu_{ep}E \qquad \ldots(4.5)$$

where, μ_{ep} is the electrophoretic mobility of the solute molecule and E is the magnitude of the applied electrical field. The electrophoretic mobility, μ_{ep} can be expressed as

$$\mu_{ep}=q/6\pi\eta r \qquad \ldots(4.6)$$

where q is the charge of solute molecule, η is the viscosity of the feed, and r is the hydrodynamic radius of the solute molecule.

Electro-ultrafiltration involves the application of an electric field perpendicular to the surface of the membrane in tandem with pressure-driven tangential flow for the separation of mixtures of charged biomolecules such as proteins/peptides.[194–196] Figure 4.4 a depicts a general schematic of a crossflow electro-ultrafiltration module. The electric field acts as an additional driving force for solute transmission along with applied pressure inside the membrane module.[194,197] The electric field also facilitates selective separation based on differences in electrophoretic mobility of the solutes.[198] Based on eq (iv), increasing the electrophoretic velocity is expected to improve the selectivity of separation. Naturally, highly charged moieties in the feed will tend to have higher electrophoretic velocity.

Application of an electric field also reduces the protein (solute)-membrane interactions.[198] A crossflow electro-ultrafiltration module was proposed by Oussedik *et al*. to reduce global membrane resistance by combining the turbulence induced by the formation of oxygen bubbles (formed from electrolysis of water at the electrode) near the membrane along with the electrophoretic movement of the proteins or other solutes under an electric field.[195] Application of pulsed electric fields across the membrane results in discontinuous electrophoretic mobilities of the solute molecules that are not in tandem with their convective movement.[199] This disrupts the convective accumulation of solute molecules at the membrane surface. Consequently, the

boundary layer thickness is lowered, thereby reducing the solute-induced filtration resistance. Similar to the application of electric field, ultrasonication disrupts the boundary layer and leads to increased flux through UF membrane.[200] However, it is not clear whether these approaches (application of electric field and/or ultrasonication) take into account, the changes in temperature (and therefore, viscosity of the solvent) due to localized heating (resistive heating and/or cavitation) that can significantly increase permeate flux.

Electrodialysis is a membrane-based separation process that utilizes an applied electric field to drive ions through ion-selective membranes. It is in contrast to electro-ultrafiltration as hydrodynamic pressure does not play a role in this form of separation. The modern electrodialysis approach was developed in the 1930s and has been employed for industrial scale desalination, deacidification of fruit juices and demineralization of whey.[201] It relies on a multicompartment apparatus that has alternating anion and cation exchange membranes. An advancement of the approach integrating UF membranes and termed electrodialysis with ultrafiltration membranes (EDUF), is a patented technology developed by Bazinet and co-workers (2005, WO2005082495A1). The EDUF setup consists of an array of UF and ion exchange membranes stacked together in a conventional electrodialysis cell (Figure 4.4 B).[202,203] It facilitates simultaneous separation of positive and negatively charged molecules and has been extensively characterized for protein and peptide separation (Figure 4.4 B).[169,203–206] In addition to charge-based separation, a recent study demonstrated simultaneous separation of different molecular weight fractions by stacking multiple membranes in the electrodialysis system.[207] The EDUF technology shows good potential for the food industry, with examples showing several different raw matrices being used for the separation and recovery of bioactive molecular fractions.[169,208] EDUF was able to overcome some of the fouling issues associated with conventional pressure-

based processing as solute-based concentration polarization is significantly reduced with electrically driven flow.[169] Both pH[209] and ionic strength[205] were found to have a prominent effect on electrodialytic separation of peptides. In particular, the pH and ionic strength played a significant role in peptide selectivity, while little or no effect observed on peptide migration rate or energy consumption in an EDUF configuration.[205,209] Increasing the electric field was shown to enhance the migration rate of the molecules, however, exceeding the limiting current density (LCD, a threshold representing maximum transport of ions through the membrane) resulted in electrolysis of water which changed the pH.[210] However, variations in peptide selectivity were not observed when the applied electric field exceeded the LCD during the fractionation of snow crab protein hydrolysate.[210] As was the case for the application of pulsed electric fields that disrupt the boundary layer, corrugated membrane surfaces improved solute transport during electrodialysis via destabilization of the diffusion boundary layer of solute on the membrane surface.[211] For the simultaneous production and fractionation of bioactives using a continuous EDUF process, Doyen *et al*. combined enzymatic hydrolysis within the electrodialysis chamber and electromigration of released peptides. EDUF is able to provide higher separation efficiencies while maintaining high recoveries.[204] For instance, EDUF was used to isolate β-lactoglobulin (80 kDa protein with immunostimulatory and antimicrobial properties) with efficiency comparable to ion-exchange chromatography.[204] Therefore, EDUF could overcome some of the fouling problems observed with conventional pressure-driven processes while also facilitating selective separation under high electric fields. However, EDUF-fractionated products might still require additional cleaning steps to remove salt components (KCl and NaCl, or other electrolytes used during processing) present in the separated fractions.

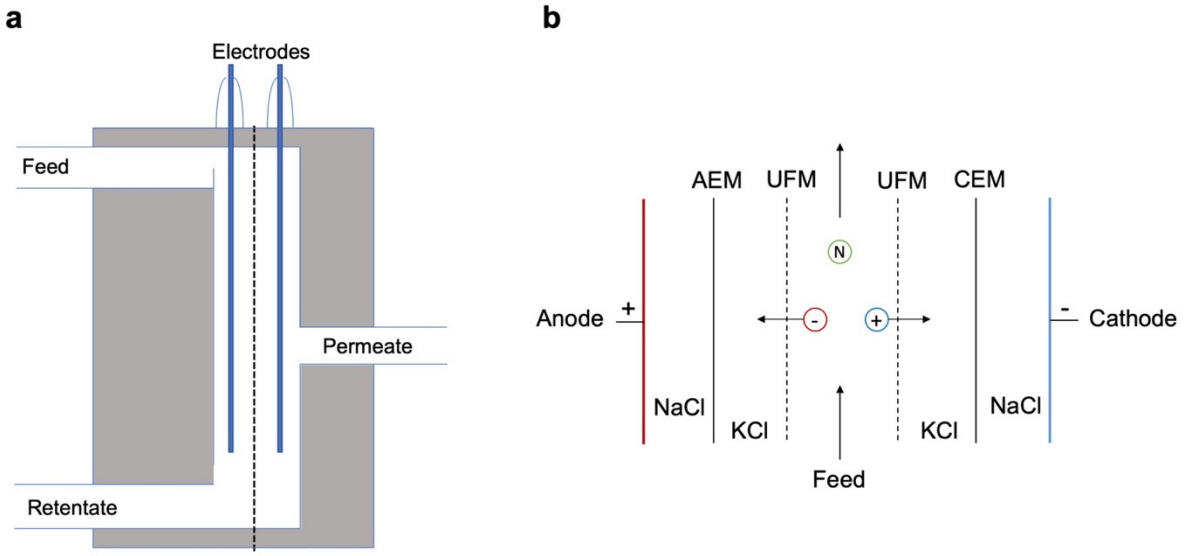

Figure 4.4: *(A) Schematic diagram of a crossflow electro-ultrafiltration module. (B) Schematic diagram of separation using an EDUF module (stacked anion/cation exchange (AEM and CEM) and ultrafiltration membranes (UFM)) (Modified from Poulin et al. (2006)).*

4.5. Downstream membrane processing of complex matrices

The previous sections described technologies that enable improved membrane separations. These approaches can be incorporated into membrane processing as a non-destructive approach suitable for food matrices. Table 4.1 highlights the latest developments in various membrane technologies used in food applications. Membrane fractionation is especially attractive for larger molecules such as proteins, or for heat labile compounds such as plant secondary metabolites (polyphenols, flavonoids etc.). Separation and recovery of the latter group of compounds should be designed based on their physicochemical properties. Another consideration to be taken account for scale-up of processing is the relative energy consumption of various approaches for separation of a given mass of material. In contrast to selectivity, which increases with lower feed

concentration as described earlier, relative energy consumption decreases with higher feed concentration.[207] The following sections describe additional parameters as that should be taken into account for processing of heterogeneous mixture of compounds from complex streams (food materials, tissue extracts etc.).

4.5.1. Electrodialytic Separation: Food-based matrices

EDUF has been successfully used to concentrate/fractionate bioactives from complex matrices such as whey protein,[114,212] snow crab protein isolate,[208] fish (herring, salmon) protein hydrolysate [207,213] and alfalfa protein hydrolysate.[169] In addition to fractionation or separation of bioactive molecules, EDUF has also been successfully utilized to enrich biomolecules in consumable products, for instance, anthocyanin enrichment from cranberry juice.[214] Sarapulova *et al*. (2018) studied the progressive fouling of anion exchange membranes during electrodialysis of wine and demonstrated that polyphenols along with polysaccharides form colloidal aggregates that deposited on the surface of the membrane.[215] π-π (stacking) interactions between phenol rings of polyphenols and aromatic groups of the membrane matrix was suggested to be primarily responsible for the adsorptive layer on the membrane. Electrodialytic membrane separation, although possessing higher fouling resistance, will still present challenges when processing complex biomatrices. Mineral salts including $CaCO_3$, $Mg(OH)_2$ and $Ca(OH)_2$ present in complex samples such as dairy products can precipitate at the membrane interface during electrodialysis.[216] Protein, peptide and amino acid fouling and its characterization during electrodialysis has been reviewed by Suwal *et al*. (2015).[217]

Pulsed electric fields disrupt the solute molecules accumulated at the membrane surface to reduce fouling. [199] Another approach called electrodialysis reversal (EDR) simultaneously changes

the electrode polarity and the flow direction to influence concentration polarization at the membrane thereby slowing down the accumulation of solute on the membrane surface. Persico *et al*. utilized permselective membranes to reduce solute accumulation via a strong hydration layer on the membrane surface.[218] Permselective membranes are made of charged resin and are covered by a very thin and highly cross-linked oppositely charged layer on its surface. Pulsed electric field,[199] electrodialysis reversal[219] and permselective membranes[218] have been described as possible solutions to the fouling problem in electrodialysis. Ion exchange membrane fouling during electro-membrane approaches is proposed to occur in the inaccessible, interstitial spaces of the membrane,[217] which makes it challenging for the cleaning process as well as experimental characterization of fouling process.

Table 4.1: *A summary of membrane technologies relevant to food applications that have been published between 2018 and 2022.*

Sl. no	Membrane technology	Area of application	Description of study	Ref
1.	EDUF	Membrane regeneration, juice and wine processing	Anion exchange membrane fouled by wine components results in increased electrical resistivity and thickness due to formation of colloidal particles at the membrane pores. Treatment of the membranes with NaCl results in salting out of the colloidal particles, with higher concentration resulting in improved regeneration of membranes.	222
			Treatment with H_2SO_4 acidified water-ethanol mixtures were more effective than NaCl solutions for regeneration of membranes.	223,224
2.	EDUF	Whey protein hydrolysate processing	Simultaneous separation of cationic and anionic peptides from whey protein hydrolysate.	119
3.	EDUF	Fish Protein hydrolysate separation	Simultaneous separation of salmon frame protein hydrolysate with three different molecular weight cutoff membranes stacked in an electrodialysis system. Cationic and anionic fractions with different molecular weight profiles were obtained as a result.	209
			Double fractionation of anionic and cationic peptides, resulting in four fractions.	215
4.	EDUF	Corn by-product: bioethanol production	Separation of acetic acid from lignocellulosic materials	225
5.	Electrodialysis with bipolar membranes	Deacidification of whey	Increased energy efficiency of acid whey deacidification with electrodialysis with bipolar membranes was possible through a preliminary demineralization step with conventional electrodialysis.	214

Table 4.1 continued

Sl. no	Membrane technology	Area of application	Description of study	Ref
6.	Membrane cascade	Oligosaccharide separation	Described the impact of temperature on yield and purity of fructooligosaccharide separation with a single-stage and three-stage membrane cascade.	184
			Optimizing separation of three different fractions of fructooligosaccharides by using multiple membrane cascade configurations and operating parameters (pressure, membrane size) which improved the fractionation and purity.	185,186
7.	Functional membranes	Processing cheese whey	Preparation of mixed matrix membranes that incorporated metal oxides for processing cheese whey effluent, which resulted in higher flux and lower fouling.	226
8.	Ultrafiltration	Skim milk	Membrane processing at higher temperatures resulted in higher rates and magnitudes of irreversible fouling despite higher flux.	227
			Membranes with higher hydrophobicity and polarity results in increased susceptibility to protein adsorption to the surface.	228
9.	Ultrafiltration	Whey protein hydrolysate	Peptide fouling was evaluated on fifteen different membranes. Membrane roughness contributed significantly to fouling.	229

4.5.2. Conventional filtration: Food-based matrices

Membrane processing of by-products from industries such as dairy, beverages (fruit juices) or fish processing can result in biofilm formation and the resulting fouling is also a major concern.[220] As mentioned earlier, reduced bacterial cell adhesion observed in functionally modified membranes with increased hydrophilicity. However, increased contact times results in increased cell adhesion even on the modified membranes.[221] Protein unfolding (bacterial cell surface proteins) at the membrane surface and stabilization as a result of hydrophobic interactions with the membrane matrix was determined to be the primary basis of cell adhesion.[221] The biofilms are resistant to cleaning cycles and can form irrespective of membrane type or surface chemistry.[220] Comprehensive examination of processing parameters and fouling behavior is essential for membrane processing of complex matrices. For instance, skim milk ultrafiltration at higher temperatures resulted in higher magnitudes and rates of irreversible fouling despite the higher flux that is associated with increased permeability.[222] When applied to isolate polysaccharides from plant extract, UF demonstrated the highest separation performance relative to those obtained through gel permeation chromatography or ethanol precipitation.[223] Optimization of transmembrane pressure, flow rates, and cut-off/pore size to maximize permeate fluxes of plant extracts is the routinely used approach.[171,223] Alternatively, several studies report the filtration performance and optimization as applied to rather simple protein/oligosaccharide mixtures (binary/multiplexed).[194,224,225] Binary or multiplexed mixtures (proteins or oligosaccharides) are consistently used to understand the basis for fractionation of complex matrices (such as cheese whey from dairy industries), rather than complex extracts.[194] More studies are necessary where process optimization and characterization for membrane filtration are carried out with samples

representative of the complexity of these streams. The dairy industry is a prime example for pioneering the development of membrane technologies. Multistage filtration systems are designed in dairy processing based on the target application, whether it be developing cheese from unfiltered milk or fractionating whey protein concentrates to derive functionally important molecules such as lactoferrin.[226] An additional processing step is the utilization of NF modules for separation of lactose, which also has an application in fermentation procedures as a carbon source. As mentioned earlier, demineralization of whey protein concentrate can also be carried out using electrodialysis.[227] These applications demonstrate that membrane processing can be designed for complex matrices based on the demands of yield and purity. Industrial scale filtration modules for size-based separation tend to use ceramic/stainless steel filtration systems rather than polymeric membrane-based filters for enhanced lifetime due to better chemical resistance and higher-pressure tolerance. However, membrane filters remain important in fractionation of macromolecular solutes, and especially for narrow molecular weight ranges for enhanced fractionation of biomolecules such as oligosaccharides and proteins.

4.6. Concluding remarks and future perspectives

Downstream processing of food-based substrates in its current form is a complex separation process which dominates production costs of bioactive compounds. Limitations of conventional separation processes have hampered the development of economically viable food products that are accessible to the general consumer. Innovations in downstream processing can lower production costs of new food products or ingredients while enabling the development of novel bioactives, nutraceuticals and functional foods. Developments in technologies such as membrane cascades, functional membranes and electro-filtration systems and their application in tandem, have the potential to enable the effective separation of macromolecular solutes present in

complex feed systems. Food membrane processing poses unique challenges resulting from high foulant feed streams and target biomolecular fractions which sets it apart from membrane applications in the environmental technology sector and water treatment. These technologies are leading towards multistage downstream processes that operate at high throughput in a continuous manner to facilitate the fractionation and purification of molecules of interest from complex substrates at scale with high recoveries. Managing membrane fouling and degradation while improving upon current methods of membrane cleaning and regeneration are essential for feasible applications of this technology in an industrial setting. Additionally, modifying operation parameters such as applied electric fields, pressure, temperature, feed flow and pH along with developments in novel charged membranes has the potential to improve upon currently available membrane technologies to reduce fouling while improving selectivity. Models that predict the behavior of various components and factors during membrane processing enable widespread and rapid optimization to facilitate tailored separation of compounds based on properties such as size and charge using these technologies from a variety of feedstocks.

Food-based bioproduct development is an area which can benefit immensely from adapting membrane technologies to suit different matrices and separate a wide variety of biomolecules. Additionally, evaluation of separation technologies needs to be carried out with complex samples that are representative of real-world heterogeneity in addition to model biomolecule solutions or mixtures. Future trends in adapting and utilizing membranes in food and bioproducts are predicted to revolve around the innovations that contribute towards development of true continuous downstream processing approaches that can lower costs and enhance recovery, selectivity and throughput.

CHAPTER 5

Selectivity of peptide separation with electrode-assisted ultrafiltration of BSA peptic hydrolysate

Abstract

Downstream processing that combines an electrical field and ultrafiltration was used for the fractionation of peptides to evaluate separation efficiency. Mechanism of peptide fractionation still remain unclear. To gain a better understanding of the peptide separation during electrode-assisted ultrafiltration (EUF), the objective of this work was to evaluate the impact of processing parameter on the separation of a protein hydrolysate generated from Bovine Serum Albumin. Peptide spectral matches along with sequence data from LC-MS/MS data were compared between the feed and permeate fractions from EUF processing. Influence of peptide properties such molecular weight and isoelectric points on separation was assessed here. Higher feed pH (7) clear imparts a higher selectivity for separation of cationic peptides during EUF operation.

5.1. Introduction

The heterogenous nature of peptides generated from naturally derived protein sources necessitates fractionation and purification steps prior to bioactive product formulation. Several studies have shown that low molecular weight peptides have more potent functional activities, including antioxidant properties, modulation of stress response, and bioavailability among other attributes.[15,25,146] Membrane filtration has consistently been described to enrich fractions of a designated peptide size range for improved functionality. Charged peptides, including those containing basic or acidic amino acid residues, are also implicated in a variety of bioactivities such as antioxidant,[85] antihypertensive,[151] anticancer,[228,229] and antimicrobial activity.[230] The net charge of a peptide is dictated by the immediate local environment (pH) along with respective isoelectric point (pI). Peptides with pI above 7 can be considered cationic, since most physiological conditions or food systems are neutral or slightly acidic. Chromatographic approaches can separate peptides based on size (size exclusion chromatography), polarity (reversed phase liquid chromatography) and charge (ion exchange). However, given the cost associated with scale-up of this technique, chromatography is not well suited for the development of functional food ingredients/nutraceuticals.[231]

Membrane fouling is more pronounced with the possibility of protein aggregation that contributes to poor filtration efficiency and low protein yield. Another major challenge limiting the use of membrane processing approaches for protein and peptide fractionation is its poor selectivity, especially for peptides or proteins of similar size ranges. One approach used to enhance membrane selectivity is the addition of electrodes, facilitating electrophoretic movement of charged species through the UF membrane. The polarity of the electric field can be chosen to select the desired charged products from a complex aqueous protein hydrolysate. Brisson *et al*., (2007)

demonstrated good separation efficacy using electro-ultrafiltration (EUF) for lactoferrin separation from whey protein isolate, which presented a mixture of five different proteins.[194] Similarly, Holder *et al*., (2013) and (2015) describe the behavior of six peptides derived from casein hydrolysate during electromembrane filtration.[232,233] However, limited examples of EUF exist for isolation bioactive peptides from a complex substrate. Several studies describe the selectivity of membrane processing approaches with binary/tertiary/quarternary mixtures. However, a more complete examination of the behavior of solutes in a heterogenous multicomponent mixture is essential to improve our understanding of solute behavior.

Here, work is focused on investigating the selectivity of EUF for fractionation of peptides generated from pepsin hydrolysis of bovine serum albumin. This system was chosen as an intermediate of the salmon hydrolysate, whose products are also generated from pepsin-like proteases. Though only a single protein standard is employed, the resulting pepsin digestion of BSA comprises of a mixture of more than a hundred unique peptides, varying in properties including pI, hydrophobicity, solubility and size. The present study directs as a goal to enrich small cationic peptides through EUF. The optimized fractionation conditions would then present an effective approach to isolate desirable cationic peptides from the more complex salmon protein hydrolysate.

5.2. Materials and Methods

5.2.1. Materials

Bovine serum albumin (BSA) and pepsin were purchased from Millipore Sigma (Oakville, Canada). The modified Lowry assay kit, and HPLC grade solvents were from ThermoFisher Scientific (Ottawa, Canada). Water was deionized to 15 MΩ cm (Sartorius Arium® Advance) and

then passed through a Barnsted Nanopure system (ThermoFisher Scientific (Ottawa, Canada)). Polyether sulfone (PES) UF membranes were purchased from Sterlitech Corporation, WA, USA.

5.2.2. Pepsin proteolysis

BSA hydrolysis was carried out a concentration of 20 mg/mL (1 L) in 10 mM HCl. Pepsin was added at a ratio of 1:100, enzyme to substrate. The solution was incubated at 37 °C for 2 hours. The BSA hydrolysate was then heated to 90 °C for 5 min for protease inactivation. The peptic digest was diluted using sodium acetate buffers (pH 3.0, 5.0 and 7.0) to a final concentration of 200 mM to a peptide concentration of 2.00 mg/mL (1 L).

5.2.3. EUF fractionation

Electro-ultrafiltration (EUF) optimization was carried out on a CF016A electrode-modified cross-flow filtration module (Sterlitech Corporation, Kent, WA, USA). The polyacrylic crossflow membrane module was set in plate-type configuration (schematic presented in Figure 5.1 and Appendix Figures A1 to A4) and the titanium dioxide coated electrodes were embedded and tightly fixed in the retentate and permeate sides. All EUF processing was carried out with 3 kDa polyethersulfone (PES) membranes (Synder flat sheet membrane ST-PES, Sterlitech, WA, USA) inserted into the module (Figure 5.1, B). Active membrane area for filtration is 20.6 cm^2.The filtration setup consisted of a diaphragm pump (Hydra-Cell D/G 04 M; Wanner Engineering Inc., Minneapolis, MN, USA) connected to the filtration module collecting feed from a 1 L container and the retentate recirculated back to the feed. The pressure was adjusted to 60 psi using a pressure control valve. The electrodes of the CF016A were connected to a DC power supply (382200, Extech instruments, NH, USA) and the current was adjusted to have a voltage (~10 V) across the membrane. The voltage was set with the cathode in the permeate side of the membrane. The set-up of the filtration module is described step by step with illustrations in Appendix Figures (A1 to

A4). Anode directed filtration results in the partial dissolution/disintegration of the electrode components of the filtration module, therefore the direction of applied electric field (when in use), was limited to cathode directed filtration in this chapter. Following collection, the permeate fraction was freeze dried (Labconco, MO, US) and stored at -20 °C for subsequent characterization.

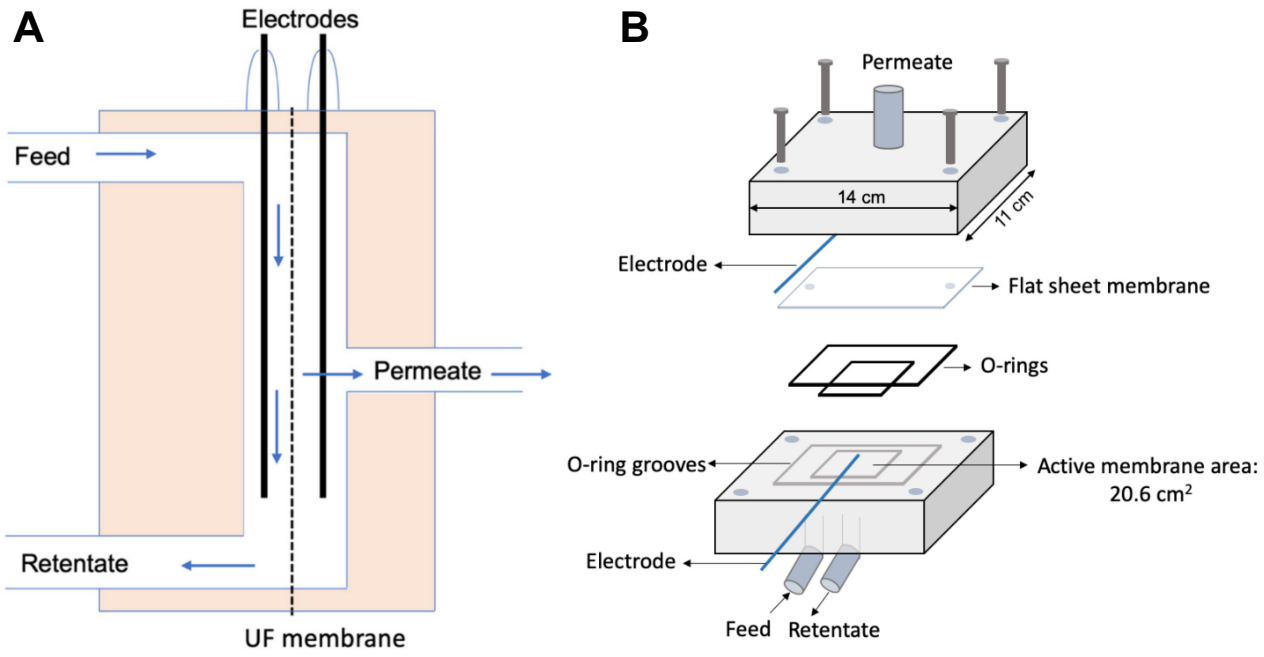

Figure 5.1: *A) Schematic representation of electric-assisted ultrafiltration cross-flow module. B) A diagrammatical representation of the organization of the EUF module*

5.2.4. LC-MS/MS analysis

BSA peptides were purified on an Agilent 1200 LC system equipped with a 1 mm × 50 mm self-packed column containing POROS® 20 R2 beads (Applied Biosystems, Carlsbad, CA) with a stepwise gradient from 5% to 85% acetonitrile in water with 0.1% TFA at a flow rate of 0.1

mL/min.[141] The purified peptide fractions were dried (SpeedVac, ThermoFisher Scientific) and resuspended in 5% acetonitrile, water with 0.1% formic acid. Eight replicate injections (10 µL) of each sample (normalized to have the same amount of protein, with 5 pmol per injection) were loaded onto a 75 µm × 30 cm self-packed column (3 µm C18 Jupiter beads, Phenomenex, CA, USA), within a PicoFrit nanospray emitter (New Objective, Woburn, MA) and separated at a flow rate of 0.25 microliters/min using a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (B): starting at 5% B, 35% by 70 min, 95% by 80 min, and lowered to 5% at 81 min using a dual capillary LC system (Agilent 1200) interfaced to an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA).[142] The LTQ operated in data-dependent mode (MS followed by MS/MS of top three peaks) with 30 s dynamic exclusion between *m/z* 400-1200. The lists of unique peptide identifications were obtained as a multiconsensus of the eight repeat injections using Proteome Discoverer v1.4 (ThermoFisher Scientific, San Jose, CA) comparing with the FASTA sequence of bovine serum albumin. A mass tolerance of 1.5 Da (precursor ions) and 0.8 Da (fragment ions) were used for searching the LTQ data and with a false discovery rate of 1%. The isoelectric points and charge states of the identified peptides were calculated using isoelectric point calculator (http://ipc2.mimuw.edu.pl/).[81] Process dependent sieving coefficients for identified peptides were obtained from the ratio of the respective normalized peptide spectral matches (PSMs).[168]

5.3. Results and Discussion

In addition to pore size, peptide fractionation during UF processes relies on additional parameters such as pH and salt concentration (ionic strength). Ionic strength and pH influences protein–protein and protein–surface (membrane and tubing) interactions which contributes to aggregation and accumulation of sample on the membrane surface, giving rise to fouling.

Therefore, proper selection of operating conditions is crucial to achieving effective separation. The processing parameters associated with EUF of BSA peptides generated with pepsin are presented in Table 5.1. The voltage was attempted to be kept constant around 10 V, although fluctuations were observed as can be seen from Table 5.1. Lower resistance at pH 5 and at pH 7, as indicated by the higher operating currents (40 mA) is predicted to occur because of higher abundance of acetate ions in the feed. The pKa of acetic acid is 4.75, below which the acetic acid-acetate equilibrium shifts towards the protonated form, which is uncharged and therefore cannot carry the current. The high pH conditions have a higher abundance of the acetate ions (15% (pH – 3), 74% (pH – 5), 99% (pH-7) thereby increasing conductivity. The change in pH during the EUF operation is a result of electrolysis of water, with OH^- ions at the cathode (reduction of water at the permeate side) increasing the pH of the permeate, while H^+ ions formed at the anode (electrode on the feed side) lowering the pH of the feed, as the retentate is continuously recirculated.

Table 5.1: *EUF processing parameters for peptic BSA hydrolysate*

Time (min)	Processing at pH 3				Processing at pH 5				Processing at pH 7			
	Voltage (V)	**Current (mA)**	**pH (permeate)**	**pH (feed)**	**Voltage (V)**	**Current (mA)**	**pH (permeate)**	**pH (feed)**	**Voltage (V)**	**Current (mA)**	**pH (permeate)**	**pH (feed)**
0	11.7	10	-	2.98	8.5	10	-	4.91	10.4	20	-	6.81
15	9.7	10	3.10	2.96	11.2	20	4.92	4.81	10.7	40	6.85	6.70
30	9.8	10	3.17	2.92	10	30	4.93	4.71	9.4	40	6.87	6.40
60	9.6	10	3.26	2.85	9.4	40	4.95	4.68	9.3	40	6.90	6.26

The operation of EUF with a feed pH of 5 resulted in non-detectable levels of peptides in the permeate as estimated by the soluble protein content measurement (estimated by modified Lowry assay). Convective flow was observed, while solute content remained negligible (replicated processing was carried out). This is hypothesized to relate to the pI associated with a large fraction of the peptides in the feed, being ~5 (depicted in Figure 5.2). This can lead to higher intermolecular interactions and aggregate formation, drastically reducing peptide translocation across the membrane. However, it is to be noted that visual observations or micrometer measurements of the membrane did not indicate a measurable detection of a fouling layer on the surface of the membrane. No aggregate formation or precipitate was observed in the feed container. Due to the low abundance of peptides in the permeate fraction of pH 5 EUF processing and given the large fraction of peptide observed near a pI of 5, the choice was made to proceed with the two other feed

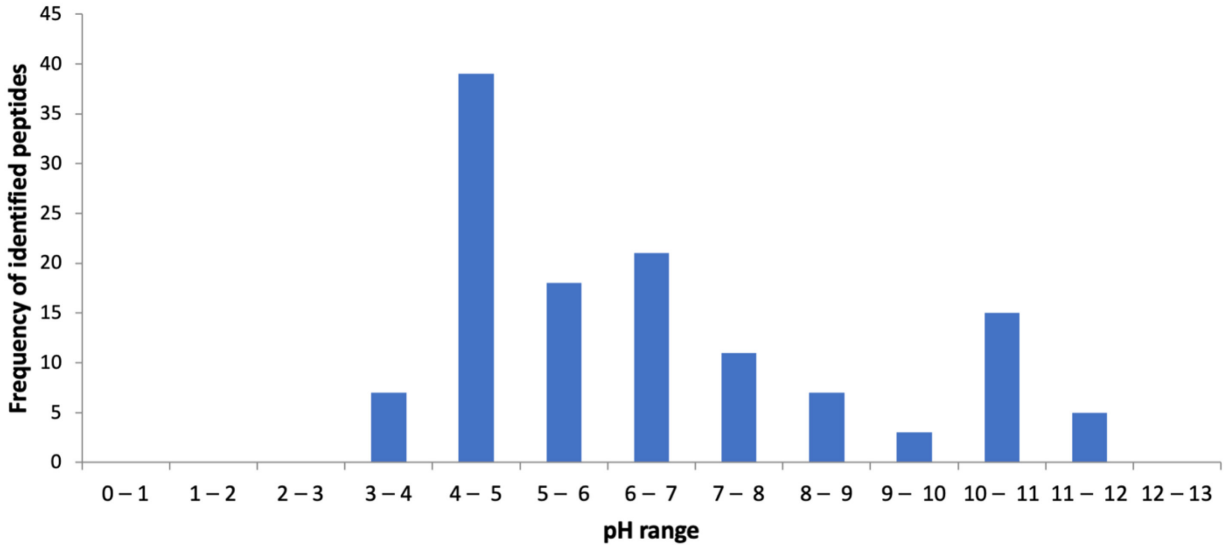

Figure 5.2: *Histogram showing the distribution of the isoelectric points (pI) for peptides identified from the BSA hydrolysate feed.*

pH conditions (pH 3 and pH 7) and characterize the selectivity of the EUF approaches from the resulting fractions.

5.3.1. EUF fractionation and enrichment

A pepsin digest of BSA represents a complex heterogenous sample mixture. Traditionally, simple binary systems or systems containing a handful of pure compounds have been employed to evaluate the selectivity of membrane processing approaches.[181,200,226] In the pepsin-generated BSA peptide feed used in this study, bottom-up proteome analysis was able to identify 126 unique peptides through tandem mass spectrometry. Specifically, 67 and 98 peptides were respectively identified from the permeate fractions of EUF at pH 3 and at pH 7. BSA peptides in the feed were found to be highly variable in both their molecular weight distribution and isoelectric points (Figure 5.2). It is acknowledged that tandem mass spectrometry peptide sequencing is biased towards peptides in the 1–2 kDa mass range and these samples are expected to contain both larger and smaller peptides (compared to the lists generated by MS/MS analysis). Also, ionization efficiency of peptides varies, which can also introduce biases in identification of peptides. To ensure a confident identification list and to remove false positives, peptide identifications with fewer than 2 spectral matches (PSMs) were removed from the list from the multiconsensus of eight injections. The peptide properties of the lists generated from the feed as well as each EUF permeate fractions are presented in Figure 5.2. In comparison to the feed (1992± 9 Da), the permeate peptide fractions at pH 3 (1407 ± 600 Da) and at pH 7 (481 ± 600 Da) both have a significantly lower ($p<0.05$) molecular weight profile (Figure 5.3 A). The reduction in size of peptides in the permeate fraction was anticipated, given that a 3 kDa molecular weight cutoff membrane was used in the fractionation for selective rejection of >3 kDa peptides and enrichment of smaller molecular weight peptides in the permeate.

Similarly, the pI profile of the permeate fractions was observed to be significantly higher in relation to peptides identified from the feed ($p<0.05$). EUF was carried in a cathode-directed set-up and the electric field is expected to selectively enrich cationic peptides (peptides with high pI). Therefore, it is evident that the EUF processing approach was able to successfully enrich small cationic peptides in the permeate fractions. However, it is noted that these comparisons were carried out without taking account of the abundance of these peptides in the fractions.

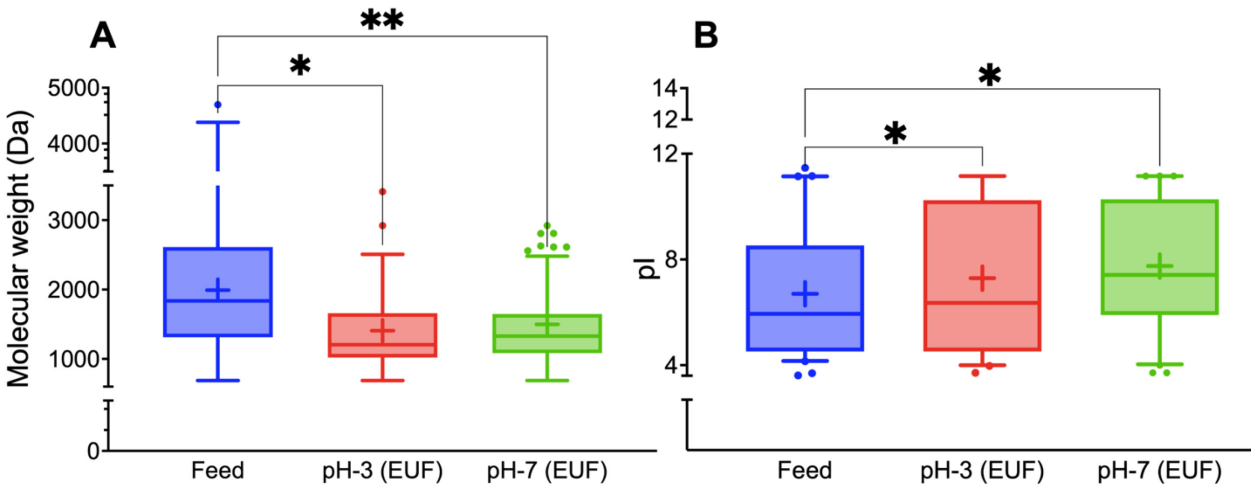

Figure 5.3: *Box-and-Whisker plots showing distribution of properties namely, (A) molecular weight (B) isoelectric point (pI) for peptides identified in the BSA feed and EUF permeate fractions processed at feed pH 3 and 7. Paired t-test was used to compare the distributions.*

*indicate significance: **$p < 0.01$, *$p < 0.05$

Spectral counting is an established approach used for semi-quantitative proteomic analysis.[234] Peptide spectral matches (PSMs) represent the number of associations that are made with a given peptide from the fragmentation spectra generated in the mass spectrometer and is

closely associated with the concentration of peptide in the sample. Figure 5.4 provides a bubble chart that illustrates the distribution of identified peptides based on their molecular weight and pI. The size of the bubble represents the number of PSMs associated with a given peptide. It is observed that the peptides in the feed are widely distributed in terms of their molecular weight (0.6 – 4.5 kDa) and pI (3.5 - 11.5). Both EUF permeate fractions are observed to have relatively lower heterogeneity based on their molecular weight and pI. The peptides in the pH 3 EUF permeate clustered at the lower mass range (Figure 5.4), indicating the enrichment of smaller peptides (0.8-1.5 kDa). Enhanced selectivity of the pH 7 EUF is evident from the larger bubbles of low molecular weight peptides at pI >8, in the permeate collected from (Figure 5.4). The following section describes the estimation of sieving coefficients and selectivity associated with the membrane separation of BSA peptides using EUF.

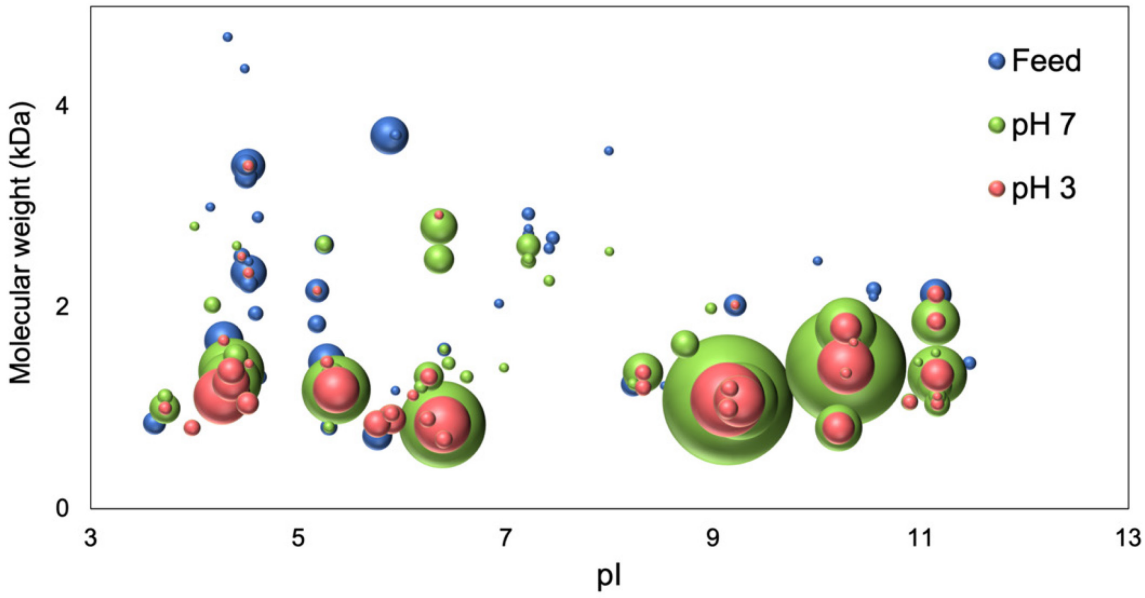

Figure 5.4: *Bubble plots showing distribution (based on pI and molecular weight) of peptides identified in the BSA feed and EUF permeate fractions processed at pH 3 and pH 7. The size of the bubbles determined by the number of PSMs (peptide spectral matches).*

5.3.2. EUF processing: sieving coefficients and selectivity

The observed sieving coefficient, S_o, of a membrane separation is determined based on the ratio of a given peptide's concentration in the permeate to that of the feed.[168] In the present study, the ratio of normalized PSMs (of a given peptide within the fraction (permeate / feed) to represent the sieving coefficient (S_o). With electrodialytic processing as described by Doyen *et al*. (2013),[77] a similar parameter, termed peptide migration rate, was determined based on the ratio of LC-UV peak area for the peptide in the permeate relative to the feed. Table 5.2 lists the top 10 most abundant peptides in each fraction along with their properties (charge at neutral pH, molecular weight) and sieving coefficients. EUF operation at pH 7 is observed to have higher S_o values compared to EUF operation at pH 3. At higher feed pH, the peptides with a net positive charge (higher pI) and can translocate across membrane as a result of the electric field. In contrast, it is evident from Table 5.2 that peptides with a net negative charge tend to have a lower S_o, especially in pH 7 operation of EUF. S_o reported in the present study for cationic peptides (in both EUF conditions) were observed to be much higher compared to a previous report by Doyen *et al* (2013).[77] S_o after 60 min of processing estimated in the present study is 10-100 fold than that the migration rate for cationic lactoglobulin peptides after 240 min of processing.[77] High sieving coefficients observed with the EUF approach is an indication of the enrichment of small cationic peptides. However, further impact of EUF processes for enrichment of peptides can be quantitatively assessed with selectivity (section 4.3.3).

Table 5.2: *List of most abundant peptides in each fraction their associated characteristics*

Feed			**EUF fraction (pH 3)**				**EUF fraction (pH 7)**			
Peptide sequence	Charge at pH 7	MW (Da)	Peptide sequence	Charge at pH 7	MW (Da)	Sieving coefficient	Peptide sequence	Charge at pH 7	MW (Da)	Sieving coefficient
WSVARLSQKFPKAEF	2.0	1794.3	SQKFPKAEF	1.0	1081.66	10.0	SQKFPKAEF	1.0	1082.55	30.6
SQKFPKAEF	1.0	1081.6	ASIQKFGERALKA	2.0	1419.09	10.1	ASIQKFGERALKA	2.0	1418.88	44.6
LYYANKYNGVF	1.0	1351.6	VEVTKLVTDL	-1.0	1116.74	5.8	FVEVTKL	0.0	835.46	10.9
PFDEHVKLVNELTE	-2.9	1669.8	FVEVTKL	0.0	835.49	4. 7	KVPQVSTPTL	1.0	1069.61	26.0
YYANKYNGVFQECCQAEDKGACLLPKIETMR	-0.1	3715.0	KVPQVSTPTL	1.0	1069.71	11.6	HVKLVNELTE	-0.9	1181.60	7.3
LQQCPFDEHVKL	-0.9	1456.9	HVKLVNELTE	-0.9	1181.67	3.4	EKLGEYGFQNAL	-1.0	1368.66	3.2
EKLGEYGFQNAL	-1.0	1368.7	EKLGEYGFQNA	-1.0	1254.73	5.2	WSVARLSQKFPKAEF	2.0	1794.08	1.7
IKQNCDQFEKLGEYGFQNAL	-1.0	2345.3	TRKVPQVSTPTL	2.0	1327.83	6.0	EKLGEYGFQNA	-1.0	1256.43	13.0
LYYANKYNGVFQECCQAEDKGACLLPKIET	-1.1	3411.5	AWSVARL	1.0	802.43	-	TRKVPQVSTPTL	2.0	1326.83	18.7
ASIQKFGERALKAWSVARL	3.0	2131.5	WSVARLSQKFPKAEF	2.0	1794.29	0.5	IVRYTRKVPQVSTPTL	3.0	1859.03	12.5

Selectivity between two different solutes during membrane separation is ratio of their S_o.[168] To discuss the effectiveness of the EUF processing approach in this study two distinct types of selectivity will be described, namely size selectivity and cationic (charge) selectivity. Size selectivity was determined based on the ratio of the sieving coefficient of any given peptide to the mean sieving coefficient of all larger molecular weight peptides in the fraction (>2 kDa). Figure 5.5 A and B shows the size selectivity trends with respect to peptide molecular weight. To reduce the noise associated with the molecular weight peptide selectivity data, the selectivity data was approximated to five bins: 0-0.5, 0.5-1, 1-1.5,1.5- 2 and 2-2.5 kDa. Peptide size selectivity indicates preferential translocation of smaller peptides in the feed across the membrane into the permeate in both conditions. The slope of the regression (Figure 5.5, A, B) is lower in the case of size selectivity of EUF at pH 7 (-11.8 ± 3) compared to EUF at pH 3 (-2 ± 0.4). This is indicative that predominance of size-based separation is reduced during the cationic directed EUF processing at higher pH as it would be at lower pH. A contributing factor to the higher size selectivity is the expected predominance of net positively charged (cationic) peptides in the slightly acidic feed at pH 3. Similarly, assessment of the influence of selectivity based on charge i.e., preferential translocation of cationic peptides during EUF will further help understand the factors in play during EUF fractionation.

The cationic selectivity of the EUF approach was determined here with the ratio of the S_o of any given peptide with that of the mean of the sieving coefficients associated with all negative/neutral peptides in the fraction (charge state ≤ 0 at pH 7). Cationic selectivity data from both EUF processing approaches were categorized into the charge state of peptides: -2, -1, 0, +1 and +2 (at neutral pH). The cationic peptide selectivity of the EUF approach is depicted in the linear regression models of box and whisker plots in Figure 5.5, C and D. This is the first

quantitative estimation and characterization of the relationship between membrane selectivity and charge state of the peptides for membrane-based processing approaches. The cationic selectivity and relationship to peptide charge (slope of linear regression; Figure 5.5 C, D) of EUF operation was estimated to be much lower than the size selectivity (Figure 5.5, A, B). This was expected as ultrafiltration is primarily a molecular size-based processing approach. It is also observed that EUF carried out at pH 7 has a higher slope of regression (0.46 ± 0.2; Figure 5.5, C) and r-squared value (0.65) compared to operation at feed pH of 3 (slope=0.31 ± 0.2, r^2=0.38; Figure 5.5, D). The positive correlation and positive slope observed in Fig 5.5 C and D is indicative of the increase in selectivity of the EUF approach with cathode placed in the permeate side for more cationic (apparently charged) peptides. Therefore, cationic selectivity trends of EUF processing feeds with varying pH is evidence of the ability of EUF fractionation to enrich cationic peptides during processing complex samples. In contrast to our expectation, the impact of feed pH EUF on selectivity remained inconclusive from the results.

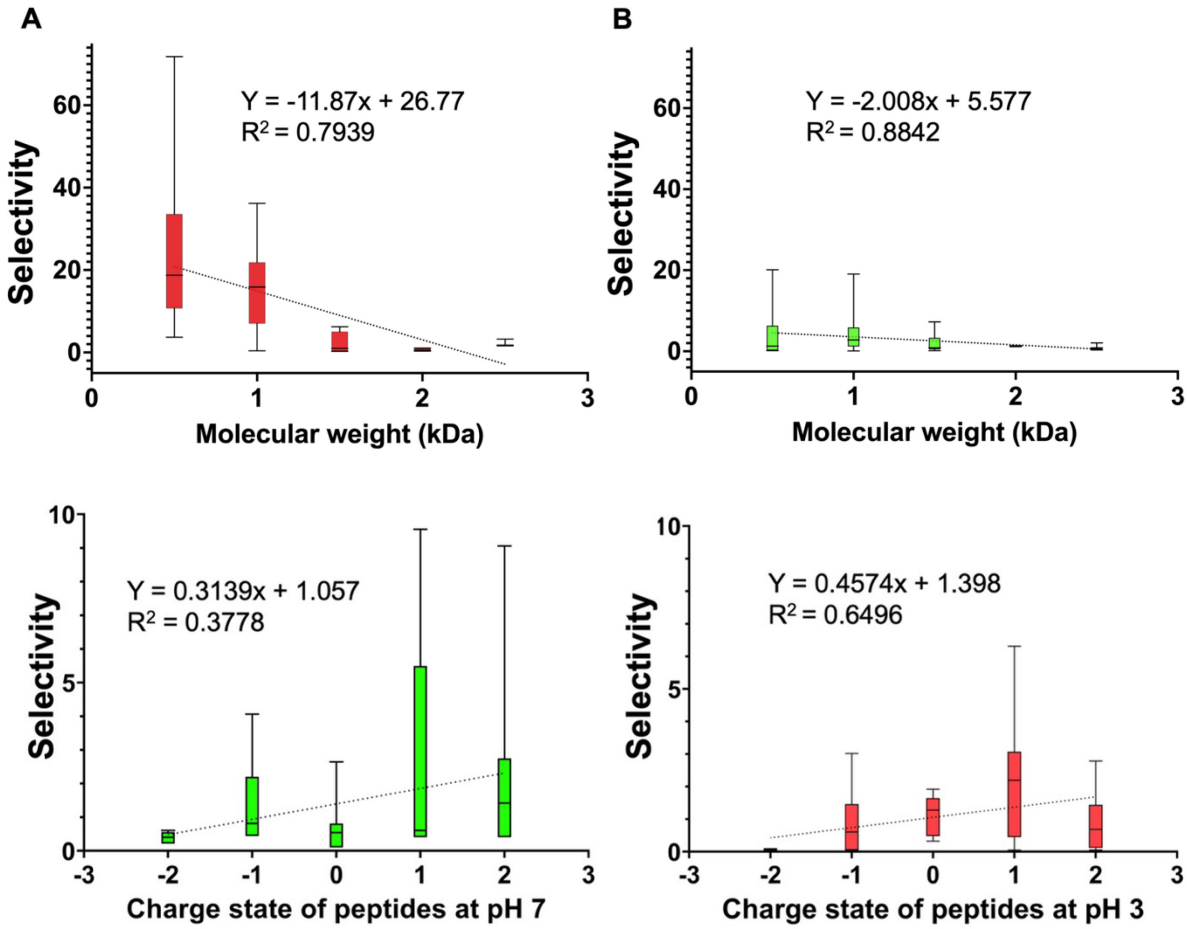

Figure 5.5: *Linear regression models of box and whisker plots associating selectivity of the EUF process with (A) Molecular weight of the peptides identified in the pH 3 EUF permeate, and (B) Molecular weight of the peptides identified in the pH 7 EUF permeate. (C) Charge state of the peptides identified in the pH 3 EUF, (D) Charge state of the peptides identified in the pH 7 EUF permeate, Slope is presented in the regression equation along with regression correlation coefficient.*

5.4. Conclusion

The presented work describes a strategy for enrichment proteins and peptides using electro-ultrafiltration. Within the constraints of operational space for the EUF module, the combined effects of pressure and voltage facilitates the enrichment of peptides based on size and charge during processing. Peptides in the 1-2 kDa range with higher pI can be efficiently enriched in the permeate using electro-ultrafiltration. This study provides a breakdown of peptide characteristics that contribute towards higher selectivity and an enhanced understanding of peptide behavior during membrane processing approach that is capable of selective enrichment of low molecular weight and charged components. Comparable charge and size selectivity of the EUF processing irrespective of feed pH as a parameter, indicates the versatility of this separation approach, especially for food matrices.

CHAPTER 6

Electrode-assisted ultrafiltration for selective enrichment of cationic and anionic peptides from Atlantic salmon protein hydrolysate generated by bacterial fermentation

Abstract

Fractionation of peptides derived from food ingredients is expected to enhance bioactivity. In this regard, membrane filtration can deliver scalable, high throughput processing of diverse feed materials. Membrane filtration alone offers limited selectivity according to molecular weight, with issues of fouling in the case of complex feed substrates. To improve selectivity and reduce fouling, electrode-assisted ultrafiltration (EUF) was used to fractionate Atlantic salmon protein hydrolysate (SPH) sourced from processing by-products through microbial fermentation. The changes in relative abundance of amino acids and molecular weight distribution using EUF of SPH were investigated along with the impact of these changes on functionality. Cationic enriched fractions were found to have higher abundance of basic amino acid residues (Lys, Arg, His) and exhibited higher metal chelation ability (118.4 ± 0.4 EDTA μM eqv.) compared to the original hydrolysate (27.9 ± 9 EDTA μM eqv.). While challenges exist for universal adoption of the EUF technology, further developments in this regard will provide the food industry with several attractive options for refined functional ingredients from bioproduct processing.

6.1. Introduction

Isolation of proteins from marine by-product tissues tends to involve hydrolysis using enzymatic or microbial processing. Since the hydrolysis products carry a generally regarded as safe (GRAS) designation, they can easily be used for animal feed, pet food applications, or for human consumption.[8,30,137] Atlantic salmon is especially relevant as one of the most valuable aquaculture species worldwide. In Canada, salmon accounted for 63% of all aquaculture production by weight at nearly 120,000 tonnes produced in 2019, capturing 74% of the aquaculture economy, and accounting for nearly $1 billion in revenue.[16] Depending on processing approaches, the salmon-derived peptides can provide enhanced functional activity. Atlantic salmon by-products have therefore been used as precursor material for a variety of functional peptide formulations.[30,51,135,235–237] Prior work carried out the Verschuren Centre found protein hydrolysates generated from salmon viscera to exhibit unique antioxidant properties.[8] As described in Chapter 2, following fermentative hydrolysis, the resulting material naturally separates into 4 distinct phases: emulsion, hydrolysate, oil and mineral. The bioactivity of peptides partitioning into the resulting crude natural product fractions correlated with their amino acid composition whereby the abundance of specific amino acid residues has been associated with higher activity. The salmon protein hydrolysate (SPH) represents the aqueous extract consisting primarily of a complex mixture of heterogenous peptides along with soluble minerals and carbohydrates.[38] It is hypothesized that the bioactivity detected from this fraction correlates to specific peptide components. Therefore, it is a goal to develop a fractionation approach capable of enriching bioactive peptides components from the salmon protein hydrolysate.

Bioactive peptides exhibit a high diversity of chemical properties including size, charge and polarity. Consequently, product formulation from food-derived bioactive peptides necessitates enrichment or purification steps to ensure sufficient activity once incorporated into food or feed matrix for consumption. In this regard, the enrichment of peptides has previously been described to improve functionality of peptide products.[25,85,228] While liquid chromatography enables separation of peptides based on size, charge, and polarity, these approaches offer limited throughput and are not economically viable when scaled for commercial processing of food stocks. In contrast, membrane filtration affords scalable, high throughput processing, although its application is limited by low selectivity, based only on molecular weight, and the potential for membrane fouling.[231] Application of an electric field across the membrane during ultrafiltration can contribute additional selectivity, by fractionation of charged species while simultaneously reducing the accumulation of solutes at the membrane that lead to fouling. This approach, termed electro-ultrafiltration (EUF), employs a crossflow module equipped with electrodes across the membrane.

Protein fractionation of model systems (single or mixture of model proteins) has been reported in the literature, including for dairy proteins such as lactoferrin.[204,226] Electrode-assisted nanofiltration was used by Lapointe *et al*., (2014) and Leem *et al*., (2014) for enrichment of a cationic peptide from tryptic digestion of purified dairy proteins, β-lactoglobulin and casein.[238,239] A combination of ultrafiltration and ion-exchange membranes, termed electrodialysis with ultrafiltration, has been used for selective separation of anionic and cationic peptides from fish protein hydrolysates.[218,240] However, EUF separation of peptides has not been demonstrated for a complex, heterogenous substrate representative of the SPH sample employed here . In this chapter, the aqueous extract of salmon protein hydrolysate was fractionated using EUF. A combination of

approaches was used to evaluate the separation efficiency of the approach according to peptide size and charge, revealing the enhanced selectivity of EUF over conventional membrane filtration approaches. Impact of separation on functional properties was assessed using antioxidant assays, demonstrating enhanced bioactivity of EUF-fractionated SPF.

6.2. Materials and Methods

6.2.1. Materials

Salmon protein hydrolysate was prepared as described in Chapter 2 (2.2.1). The modified Lowry assay kit, and HPLC grade solvents were from ThermoFisher Scientific (Ottawa, Canada). Water was purified with a Sartorius Arium® Advance and then passed through a Barnsted nanopure system (ThermoFisher Scientific (Ottawa, Canada)). PES UF membranes were purchased from Sterlitech Corporation, WA, USA.

6.2.2. Preparation of salmon protein hydrolysate (SPH)

The salmon protein hydrolysate was passed through a vacuum filtration apparatus equipped with a 0.45 μm polyethersulfone (PES) microfiltration membrane to remove particulate material prior to UF. The clarified salmon protein hydrolysate (SPH) was collected and aliquoted to be stored at -20 °C until further use. A 250 mL SPH aliquot was also freeze dried (Labconco, MO, US) for additional analysis.

6.2.3. Fractionation of SPH (UF/EUF)

Ultrafiltration or electro-ultrafiltration (EUF) optimization was carried out on a CF016A electrode-modified cross-flow filtration module (Sterlitech Corporation, Kent, WA, USA) connected to the filtration module. a diaphragm pump operating at 35 Hz (Hydra-Cell D/G 04 M; Wanner Engineering Inc., Minneapolis, MN, USA) as described in Chapter 5 (5.2.3). Flux across the membrane was expressed as $mL.cm^{-2}.min^{-1}$ based on the volume of permeate collected between

a given period with the effective surface area of filtration being 20.6 cm^2. The collected permeate was freeze dried (Labconco, MO, US) and stored at -20 ºC for further characterization.

6.2.4. Evaluation of process components

6.2.4.1. Membrane thickness assessment

The PES membrane thickness was evaluated before and after processing with a digital micrometer (Marathon Watch Company Ltd., Richmond Hill, ON, Canada).

6.2.4.2. Soluble protein content

Samples collected at different time points were evaluated for soluble protein content as is, which was determined using modified Lowry assay kit (ThermoFisher Scientific, IL, US). A Tecan M1000 microplate reader (Männedorf, Switzerland) was used in the analysis.

6.2.5. Evaluation of peptide composition and profiling

6.2.5.1. Amino acid composition

Acid hydrolysis (6N HCl, 1% phenol; 110 ºC, 24 hours) of samples was carried out in PicoTag reaction vials (Eldex Laboratories Inc., Napa, CA, USA). The reaction vial is dried under vacuum. Precolumn derivatization of dried samples (vacuum drying followed by creation of a nitrogen atmosphere) was carried out with phenyl isothiocyanate (PITC) at room temperature in 7:1:1:1 methanol, water, triethylamine and PITC. Diluted sample/standard (2 µL) was injected on a Waters Acquity UPLC equipped with BEH C18, 2.1 x 100 mm (1.7 µm) at a flow rate of 0.5 mL/min. Reversed-phase gradient elution was achieved using a mixture of Buffer A (0.14 M sodium acetate, 0.05% triethylamine, pH 6.05, 6% acetonitrile) and Buffer B (60% acetonitrile) and signal monitored at 254 nm. Based on the injected amount (50 picomoles) and integrated areas of each amino acid in the standard, the quantities in the unknown sample were determined.

6.2.5.2. Gel permeation chromatography

SPH and fractionated samples (20 µL at 4 mg/mL) were injected on an Agilent 1260 Infinity series II equipped with GFC P2000 column (300 x 7.8 mm, Phenomenex, CA, USA) and a variable wavelength detector set at 214 nm. The mobile phase 45% acetonitrile, 0.1% TFA in water was operated at the flow rate of 0.85 mL/min. The calibration curve was prepared using Cytochrome C (12327 Da), Bovine Insulin (5733 Da), angiotensin (1046 Da) and glutathione (307 Da).

6.2.5.3. Cation-exchange chromatography

Peptide samples, namely SPH and EUF/UF fractions were injected (50 µL) to a Luna® 5 µm strong cation exchange (SCX) column 100 Å, 150 x 4.6 mm (Phenomenex, CA, USA). Flow rate was set at 1 mL/min and the composition of mobile phase A was 20 mM potassium phosphate, pH 3, while mobile phase B was 20 mM potassium phosphate pH 3 with 0.5 M potassium chloride and C was 100% acetonitrile. The gradient method used for separation is described in **Table 6.1**.

Table 6.1: *Separation gradient timetable used for the SCX separation of salmon peptide fractions*

Time (min)	Flow rate (mL/min)	A (%)	B (%)	C (%)
0	0.85	75	0	25
6	0.85	70	5	25
10	0.85	65	10	25
14	0.85	50	25	25
18	0.85	25	50	25
22	0.85	0	100	0
24	0.85	75	0	25

6.2.5.4. Proteomic analysis

LC-MS/MS analysis was carried out with samples injected with an Easy nLC system (ThermoFisher Scientific, San Jose, CA) connected to a Q Exactive HF-X (ThermoFisher Scientific, San Jose, CA) operating at 60,000 resolution in positive mode with a scan range of *m/z* 400 to 1600. Reverse-phase separation of peptides was carried out (60 min) in the nLC running at 250 nL/min with a gradient program of 3% B (0.1% formic acid in acetonitrile) at 0 min to 20% B at 18 min to 35% B at 49 min to 100% B at 51 min while Solvent A was 0.1% formic acid in water.

Peaks studio 10.6 (Bioinformatics solutions Inc., ON, CA) was used for peptide searching against the *Salmo salar* UniProtKB database (downloaded June 2021, 82272 entries) with parent mass error tolerance of 10 ppm and fragment mass error tolerance of 0.2 Da. Peptide lists were generated with database searches carried out using unspecified cleavage (no enzyme), semi-specific pepsin and semi-specific trypsin with a FDR of 1%. The peptide lists were compared using Venny 2.1 (BioinfoGP). Isoelectric points of the identified peptides were calculated using isoelectric point calculator http://ipc2.mimuw.edu.pl/.[81] GRAVY scores for the identified peptides was computed using an online tool: https://www.bioinformatics.org/sms2/protein_gravy.html.[80]

6.2.6. Antioxidant assays

6.2.6.1. Ferric reducing antioxidant potential assay

Ferric reducing capacity of SPH and peptide fractions were assessed by the method developed by Oyaizu (1986)[144] using a similar approach and has been described in section 2.2.10.

6.2.6.2. Fe(II) chelation assay

The capacity for peptides to bind Fe (II) was evaluated using the metal chelation assay developed by Boyer and McCleary (1987) with modifications by Saidi *et al*., (2014), at a peptide concentration of 4 mg/mL.[87,145]

6.2.7. C-18 Solid phase extraction (SPE) purification

Microfuge tubes were placed into a Visiprep™ vacuum manifold (Supleco) and 1 mL of sample (4 mg/mL) was passed through a C-18 Solid phase Extraction (SPE) cartridge (Hypersep™ SPE, 100 mg C18, ThermoFisher Scientific, TN USA). The SPE cartridges were washed twice with water after which the purified peptides were eluted with 0.6 mL of 75% acetonitrile. Fractions were dried prior to further analysis of antioxidant activity.

6.3. Results and Discussion

6.3.1. SPH fractionation and recovery with EUF

Application of voltage was evaluated to explore the impact on permeate flux and membrane fouling. In any membrane filtration process, the permeate flux is a significant parameter that influences the recovery of the fractionation process. The filtration apparatus was operated at a constant 60 psi, made uniform with a diaphragm pump. Lower operating pressures were attempted (<30 psi), though this resulted in highly variable flux and peptide recovery. It is speculated that this results from irregularities in membrane morphology, whereby differences in membrane thickness >5 % was observed even within a single sheet. Inclusion of an applied electric field during ultrafiltration (*i.e.* electro-ultrafiltration or EUF) has previously been shown to reduce membrane fouling. [241–244] From Figure 6.1, cathode-directed EUF, referring to the placement of the negative electrode (the cathode) on the permeate side of the membrane, maintained the highest permeate flux profile over the course of two hours. In contrast, anode directed filtration yielded a

significantly lower permeate flux over the course of filtration. This is explained by noting the impact of the applied voltage on the titanium electrode. Anode directed filtration caused an observable dissolution of the anode, whereby. the TiO_2 coating on the electrodes are eroded because of electrolytic reactions. Electrodissolution can therefore lead to an accumulation of contaminants in the permeate and retentate. This issue can be overcome in future studies by using an inert electrode such as platinum. Varying results have been reported for electrically-driven separations, resulting from complex electrolytic reactions taking place at the electrodes.[196,216,238] Anode-directed filtration is also challenged by the acidic pH of the feed (4.6) implying that a large portion of SPH peptides are cationic. These species are not assisted by the voltage gradient to traverse the membrane, thus increasing the cake layer formation on the membrane and reducing permeability. However, it is clear that cathode-directed filtration was found to provide a higher flux of the SPH feed through the membrane.

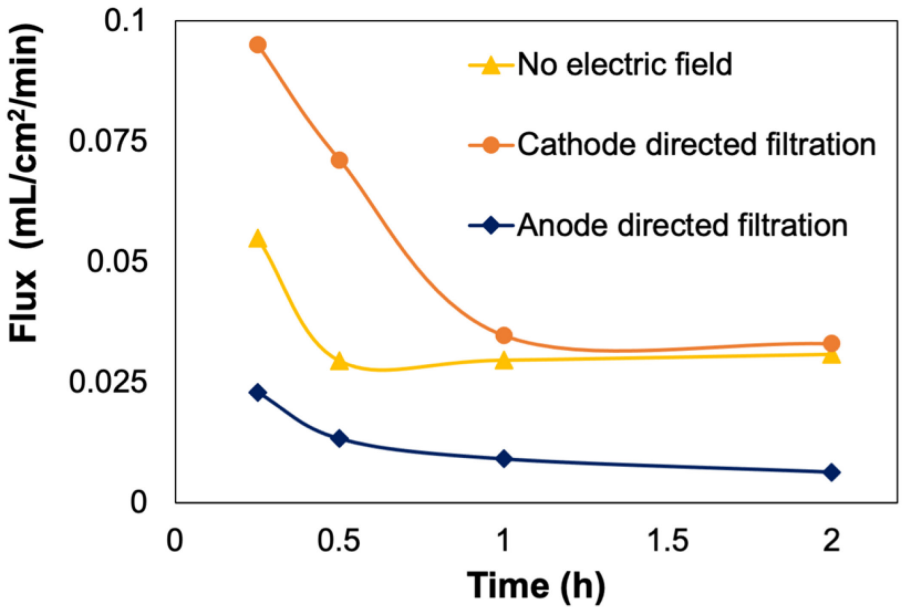

Figure 6.1: *Flux profiles of the SPH fractionation carried out by different modes of operation with/without applied electric field and direction of electric field (anode or cathode directed filtration).*

Varying the concentration of the feed was expected to impact the degree of membrane fouling, and therefore the associated permeate flux. It was noted that the desired 10 V applied across the membrane was difficult to maintain for dilute peptide solutions (<1 mg/mL soluble protein content), owing to low conductivity of the feed. The high current necessary to maintain voltage across the membrane can cause undesirable sample heating and may also exceed the limit of the power supply. While addition of electrolytes such as KCl, could maintain conductivity of lower feed concentrations during processing,[203] this would result in very high salt accumulation in the permeate, which is undesirable. Therefore, a feed concentration of at least 1 mg/mL was deemed necessary for the experimental setup.

Direct evidence of the influence of feed concentration on membrane fouling is provided through measurements of the thickness of the membrane following two hours of filtration. As seen in Table 6.2, an increase in membrane thickness correlates with the concentration of peptide in the feed solution. The high standard deviations reported reflect the variability associated with the fouling process across different regions of the membrane. Persico *et al*., (2018) observed negligeable peptide fouling (whey protein hydrolysate) on PES membranes.[240] However, the membrane cutoff in that study was 50 kDa, while the membrane processing in the current study used a 3 kDa cutoff membrane. Furthermore, membranes were not assessed in a processing approach (a true filtration experiment) as carried out in this study. Rather, the membranes were simply soaked in the feed solution to determine if peptides adhere to the surface. A clear layer of protein fouling was observed by Ng *et al*., (2018) during skim milk filtration using PES membranes as a result of adsorption/accumulation on the membrane surface.[222] In the present study, a yellowish layer was observed to be formed on the surface of the membrane after fractionation of SPH during all treatments.[240]

Table 6.2: *Fouling thickness on PES membranes after processing at different feed concentrations.*

Conc. (mg/mL)	**EUF (μm)**	**UF (μm)**
1	8 ± 5	7 ± 4
2	8 ± 7	13 ± 3
3	12 ± 6	15 ± 6

The ultrafiltration membrane thickness was variable ranging from 160-190 μm. The thickness of the fouling layer on the surface of the membrane as observed in Table 6.2, was slightly reduced at higher protein concentrations through application of the electric field. However, membrane fouling was shown to be higher in the UF processing (without voltage), demonstrating that EUF processing can employ higher feed stock concentrations, without causing substantial membrane fouling. This is demonstrated in Figure 6.2. In particular, the permeate flux with EUF at 1 mg/mL overlaps that of 2 mg/mL (Figure 6.2A), following a short equilibration period. The permeate flux was also higher in EUF at all times, and for all feed concentrations, compared to filtration in the absence of voltage (Figure 6.2B). In general, higher feed concentration decreases the flux across the membrane according to the stagnant film model.[175] Increased feed concentration leads to higher concentration polarization at the surface of the membrane, causing lower flux through the membrane.[231] Consistent with the previous observations from Figure 6.1, flux decline reduced during the initial phase of processing with cathode directed EUF. Similarly, enhanced permeate flux has been described by several studies through the application of an electrical field during ultrafiltration.[241–244]

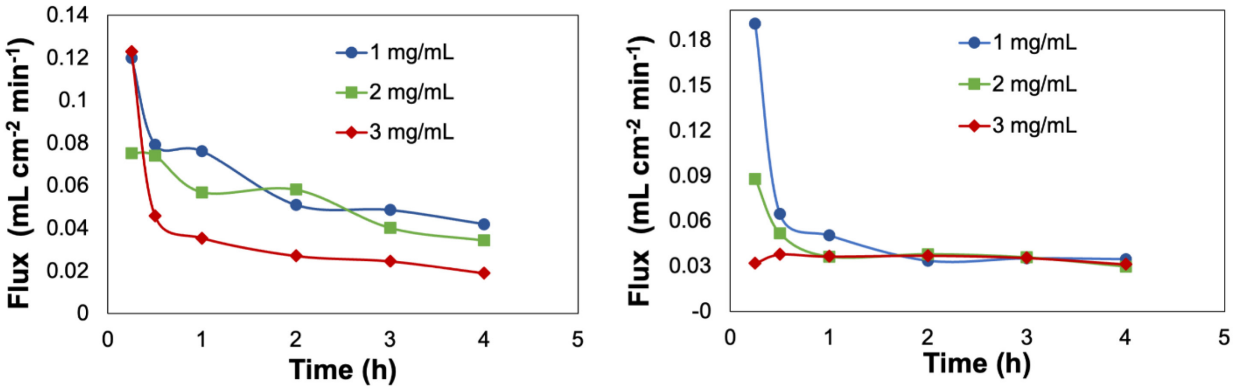

Figure 6.2: *Flux profiles of the SPH at different soluble protein concentrations, carried out (A) with the application of voltage in cathode directed filtration mode, and (B) without applied voltage.*

The soluble protein content was evaluated for samples of permeate flux collected over various time points during processing. No statistically significant difference ($p<0.05$) was observed for the soluble protein content (~4 mg/mL) determined at various time points for the permeate separated using UF (no voltage) and cathode directed filtration with a final protein concentration of 4.5 ± 0.4 mg/mL in the permeate. In contrast, anode directed filtration resulted in a lower ($p<0.05$) soluble protein content in the permeate (final protein conc. 3.5 ± 0.8 mg/mL) compared to the retentate (final protein conc. 5.6 ± 0.6 mg/mL).

6.3.2 Peptide profile of fractions: Impact of separation

Changes to the amino acid composition is an indication of selective migration of peptides into the permeate fraction during processing. Selective enrichment of basic peptides into the permeate is achieved by cathode directed EUF, facilitating translocation of cationic moieties across the membrane during EUF. Therefore, cathode directed EUF was expected to increase the

abundance of basic amino acid residues in the permeate, namely Lys, Arg and His. Figure 6.3 summarizes the relative abundance of acidic and basic amino acid residues collected in the permeate (A) and retentate fractions (B). Table 6.3 outlines the names and details associated with various fractions collected after fractionation.

***Table 6.3**: List of peptide fractions and their description*

Name	MW cutoff membrane	Mode of filtration	Fraction type
SPH	none	none	Feed
<3A	3 kDa	Anode directed EUF	permeate
<3C	3 kDa	Cathode directed EUF	permeate
<3N	3 kDa	No voltage/UF	permeate
>3A	3 kDa	Anode directed EUF	retentate
>3C	3 kDa	Cathode directed EUF	retentate
>3N	3 kDa	No Voltage/UF	retentate

As expected, cathode directed filtration selectively enriched peptides with basic amino acid residues in <3C. This is evident through a comparison of <3C to <3N, as well as to <3A. Lysine was the 2nd most abundant amino acid residue in <3C, being ~50% higher than the original feed solution, and more than 3-fold higher than <3A. Similarly, cathode-directed filtration yielded a corresponding drop in acidic amino acids in <3C. These results therefore suggest that peptide separation through EUF is influenced by the charge of the peptides. Enrichment of peptides containing basic or acidic amino acid residues is resultant of the placement of cathode or anode on the permeate side of the membrane during processing.

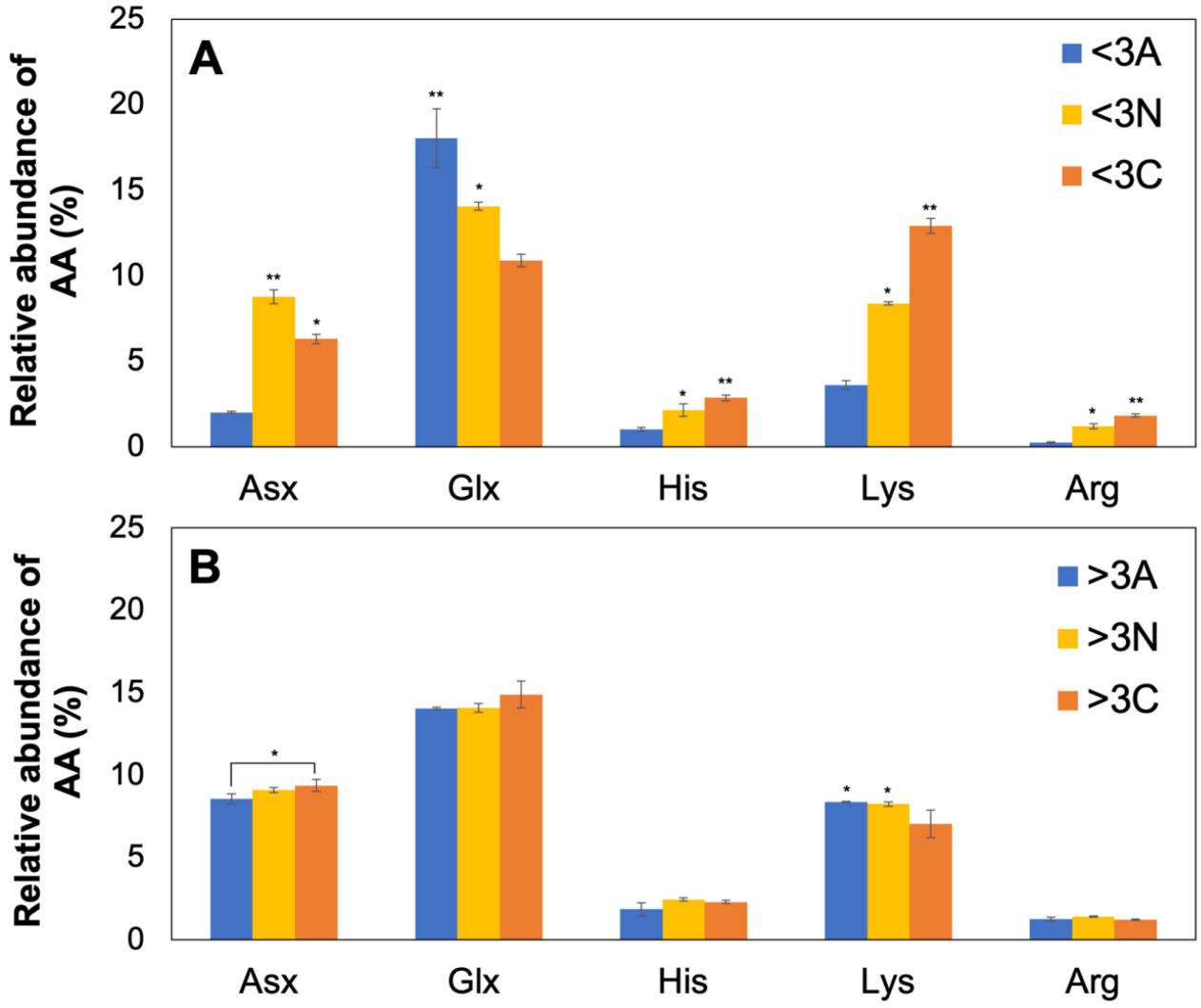

Figure 6.3: *Relative amino acid abundances of (A) permeate fractions and (B) retentate fractions generated from SPH.*

Means (bars) denoted by a different number of asterix represent significantly different means ($p < 0.05$) based on multiple comparison of means (Tukeys test) using one-way ANOVA.

The charge characteristics of peptide mixtures can also be evaluated using strong cation exchange separation. Higher retention on SCX column is an indication of more cationic peptides, or alternatively of higher mass peptides. However, higher mass peptides are unlikely since all three permeate fractions enrich for solutes below a 3 kDa molecular weight. Figure 6.4 depicts the elution profile of peptides from SPH and the permeate fractions. As seen in the figure, <3A fraction shows little evidence of highly cationic peptides, as minimal signal is observed beyond 10 min in the chromatogram. This agrees with the reduced content of basic amino acids (Figure 6.3). The chromatographic profile of the UF permeate in the absence of applied electric field is similar to the unfractionated SPF sample, indicating no preference towards selection or depletion of highly cationic peptides. Finally, the chromatogram of the <3C shows evidence of enriched cationic peptides, with a relative increase in peak area beyond 10 min compared to the unfractionated control. Together with the corresponding amino acid profiles, these results provide strong evidence to support the selective enrichment of positively charged peptides by the electro-ultrafiltration apparatus. These cationic peptides are most desired for their bioactive properties, which are evaluated further in the subsequent sections.

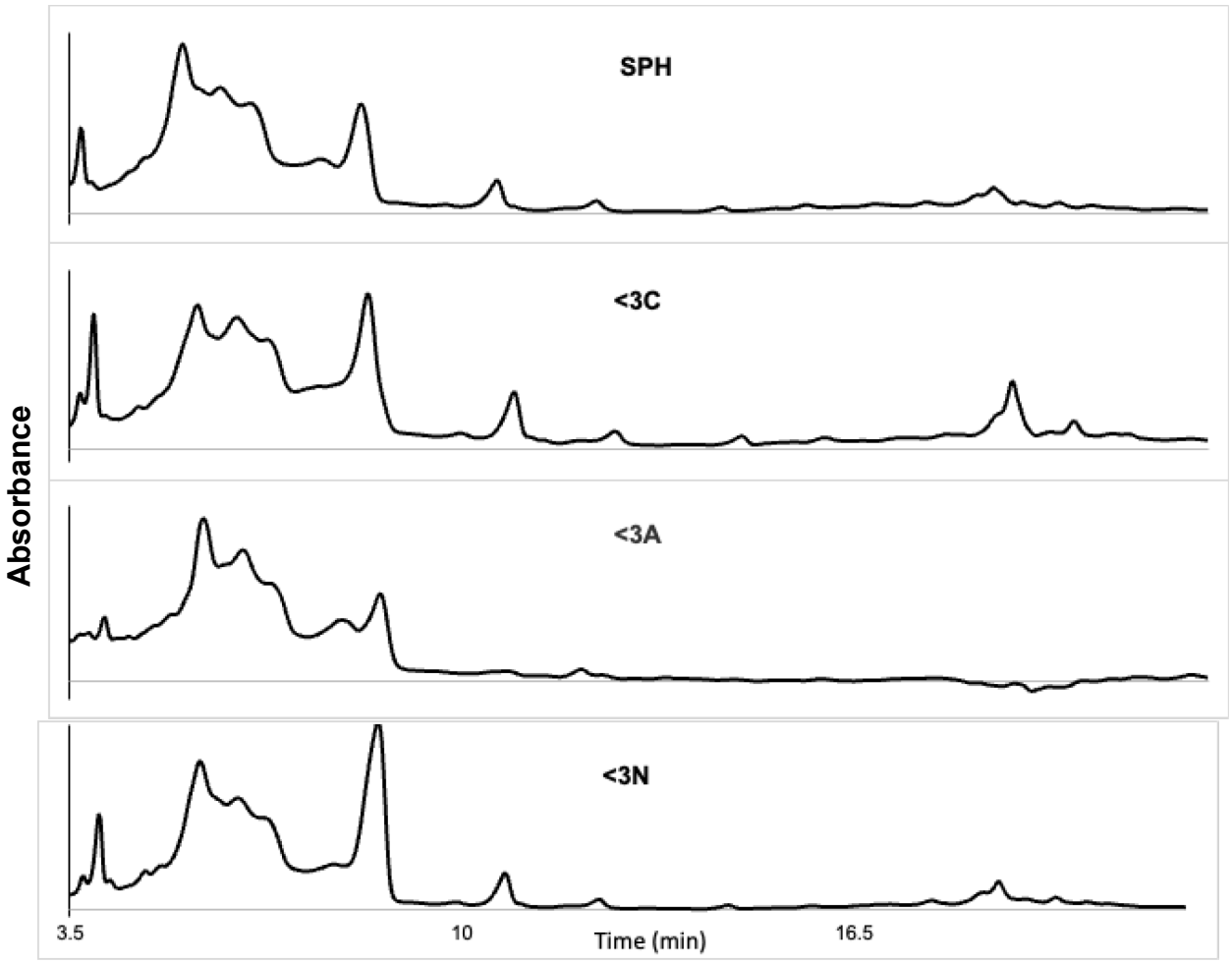

Figure 6.4: *SCX elution profiles of SPH and permeate fractions generated by EUF.*

The analytical characterization of peptides based on size was also conducted using gel permeation chromatography (GPC). A nominal 3kDa membrane should show preference towards lower molecular weight peptides in the permeate, noting that the 3kDa pore size is not an absolute cutoff. Thus, a trend tending towards smaller peptides in the permeate was anticipated. Figure 6.5 summarizes the results obtained by GPC analysis, displaying the molecular weight distribution of peptides in the permeate fractions relative to the unfractionated SPH. These chromatograms confirm the higher selectivity for low MW components by the membrane filtration process. All

permeate fractions contained a minimum 50% of <500 Da components. It was observed that each fraction retained higher MW components above 3 kDa, although the higher MW components above 5 kDa are reduced to negligible levels in the permeate fractions following fractionation. that the polarity of the applied electric field had little impact on the peptide MW distributions, which was expected. The molecular weight profiles were statistically indistinguishable for all three retentate fractions (>3N, >3A, >3A). However, in all cases, the peptides in the permeate fractions contain a greater proportion of low MW peptides. From a processing perspective, conventional ultrafiltration of the SPH does not significantly impact or enhance the chemical characteristics of the sample. Therefore, the orthogonality offered by the EUF separation to enrich charged peptides is especially valuable to this sample (SPH).

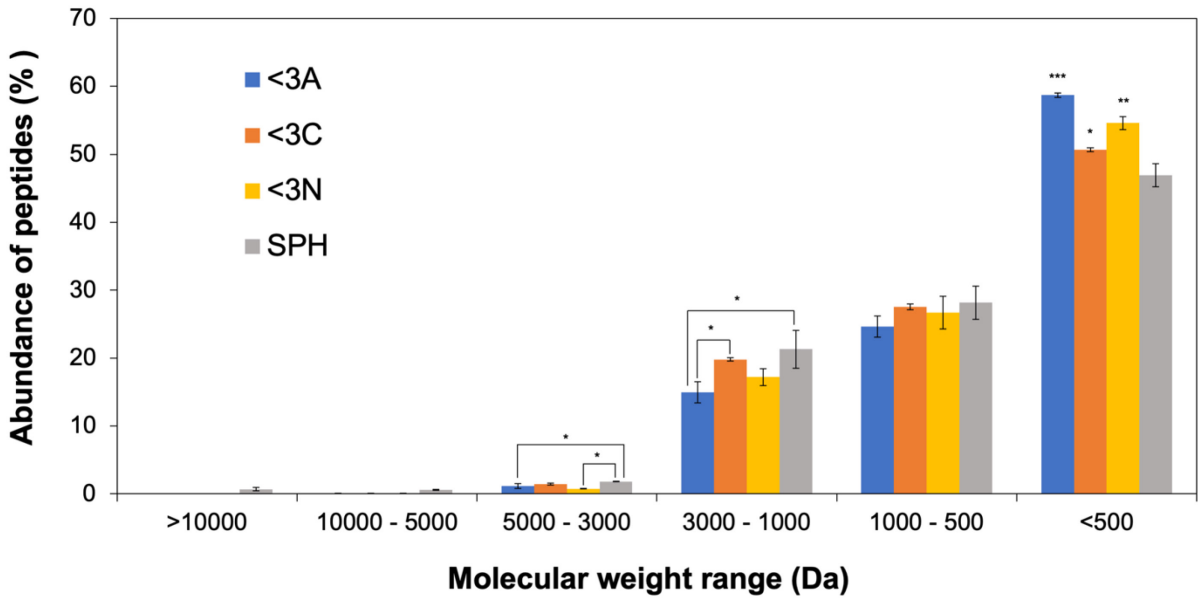

Figure 6.5: *Molecular weight profile of UF/EUF peptide fractions*

Means denoted by a different letter indicates significant differences between means ($p < 0.05$) based on multiple comparison of means (Tukeys test) using one-way ANOVA.

The peptides in the UF/EUF permeate fractions were subject to LC-MS/MS characterization. The neutral fractionation generated 105 unique peptide identifications while <3A and <3C had 79 and 65 unique peptide identifications respectively (Figure 6.6). From this Venn diagram, the <3C and <3A fractions were observed to have the least similarity based on the low overlap in identified peptides. The non-specific cleavage associated with SPH production is responsible for smaller lists of confident peptide identifications from the LC-MS/MS runs. As described in section 1.6.5, probability of confident identification decreases significantly with unspecified cleavage of proteins, thereby reducing peptide lists. Although >50,000 ions were detected in the MS analysis, confident identification of peptides remained low even though a high-resolution mass spectrometer (Q Exactive HF-X (ThermoFisher Scientific, CA, USA)) was used for the analysis. Most of the identified peptides were observed to be derived from collagen isoforms. Given the source of salmon proteins, this result was expected.

The peptide sequences were further examined to evaluate trends on GRAVY scores, MW and pI. While the comparison of permeate fractions yielded no distinguishable features for MW and GRAVY scores (not shown), a frequency distribution of pI values of peptide sequences is presented in Figure 6.6, B. It is observed that <3A has the highest abundance of acidic peptides, which would generally give rise to anionic species as confirmed from amino acid and SCX chromatography analysis. Similarly, <3N had a higher frequency of peptides identified with pI in the neutral to high pH region (pH 5-8), therefore tending towards cationic peptides. However, <3C did not follow this trend, which is speculated to be a result of the low number of peptide identifications from this fraction.

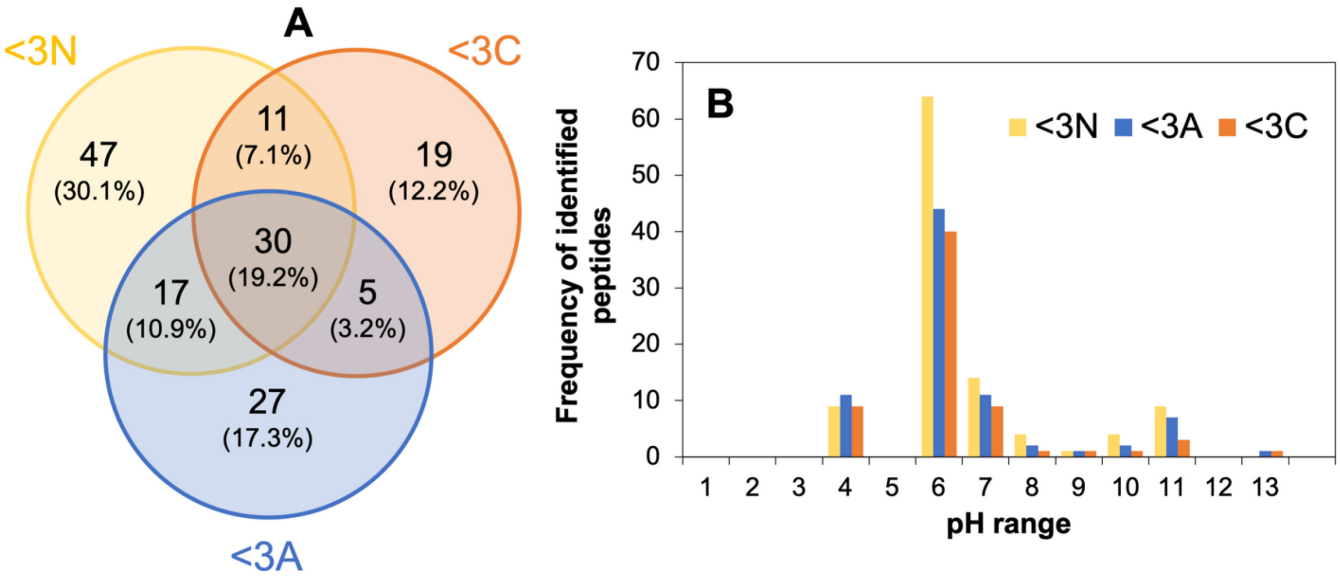

Figure 6.6: *(A) Venn diagram comparing identified peptide sequence from the three peptide fractions (B) Distribution of pI of peptides identified from the membrane processed fractions.*

The MS results corroborate the selective peptide partitioning based on charge size and charge during EUF, as described with LC-UV results earlier. Noting the detection of >50000 ions per fraction, whose sequence could not be positively confirmed, profiling the mass and charge state of the detected ions was attempted. Figure 6.7 provides histograms of the peptides from each permeate fraction according to their molecular weight and charge distribution. The MW profiles shown here are similar to those reported via GPC analysis (Figure 6.5). Similar to the SCX profiles (Figure 6.4), higher abundance of ions with +2 and +3 charge states were observed in <3C, compared to both <3N and <3A. Summarizing the results of amino acid content, LC profiles and MS characterization, it is evident that EUF enhances the partitioning of smaller MW charged (cationic/anionic) peptides into the permeate fractions.

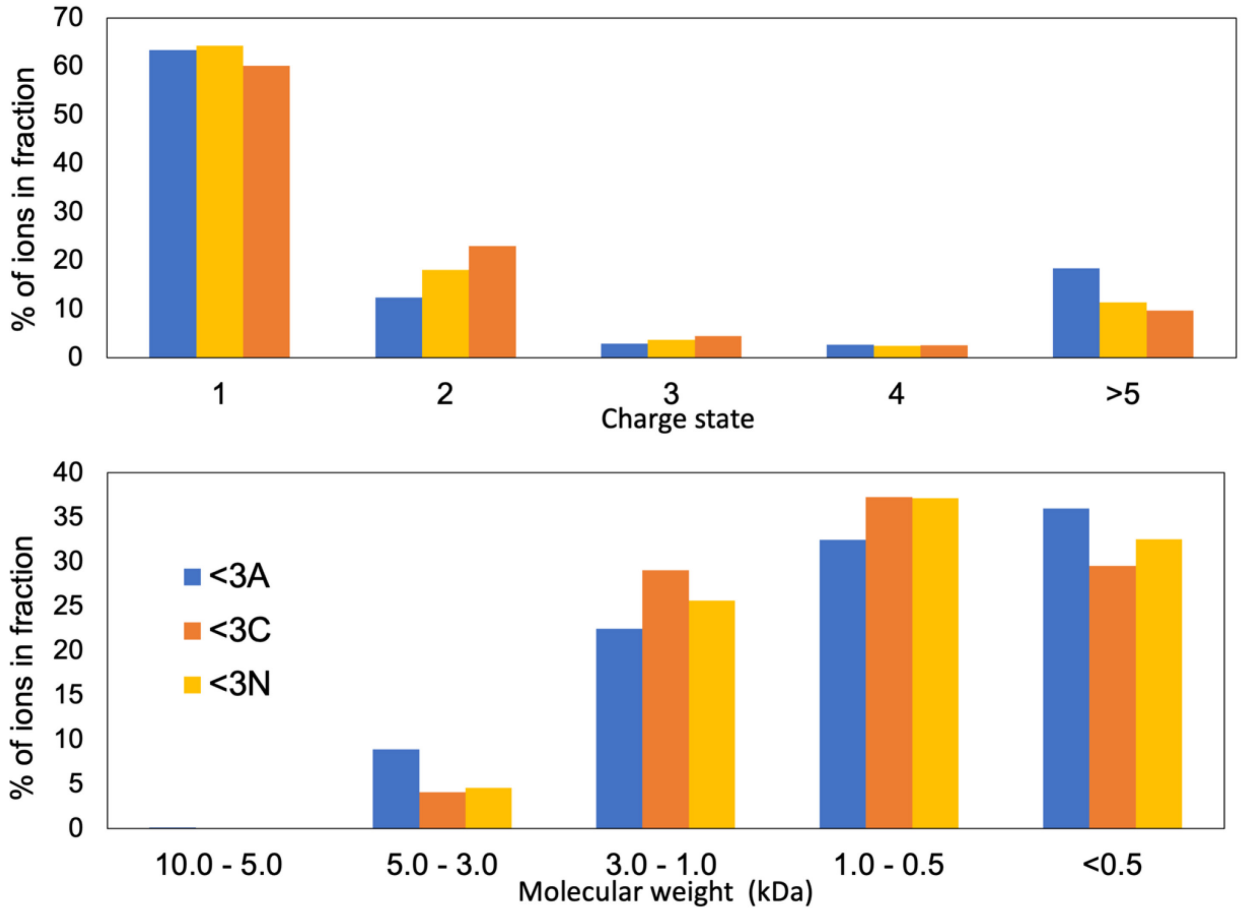

Figure 6.7: *EUF/UF peptide fraction profile on the basis of (A) charge status, (B) mass profile; as determined by LC-MS/MS.*

6.3.3. Antioxidant activity

The ferric reducing antioxidant potential (FRAP) assay measures the ability of peptides to act as an electron donor. Figure 6.8 (A) illustrates the ferric reducing capacity of salmon peptides in the unfractionated SPH, as well as the membrane-filtered peptide fractions. Previous studies have demonstrated smaller peptide fractions (ultrafiltered peptide fractions) to yield higher reducing capacity.[92] As seen here, none of the fractionated permeates displayed higher ferric reducing capacity. By contrast, the UF permeate retained in the absence of applied electric field showed significantly lower ($p<0.05$) ferric reducing capacity. Both the permeate and retentate

fractions from anode directed filtration exhibited pro-oxidant behavior with the FRAP assay. However, this result can be explained by the presence of contaminants from electrode dissolution observed during separation, which may have acted as prooxidants. Anionic and cationic trout peptide fractions derived by an electroanalytic process by Suwal *et al*., (2018) did not improve reducing capacity of the fish protein hydrolysate.

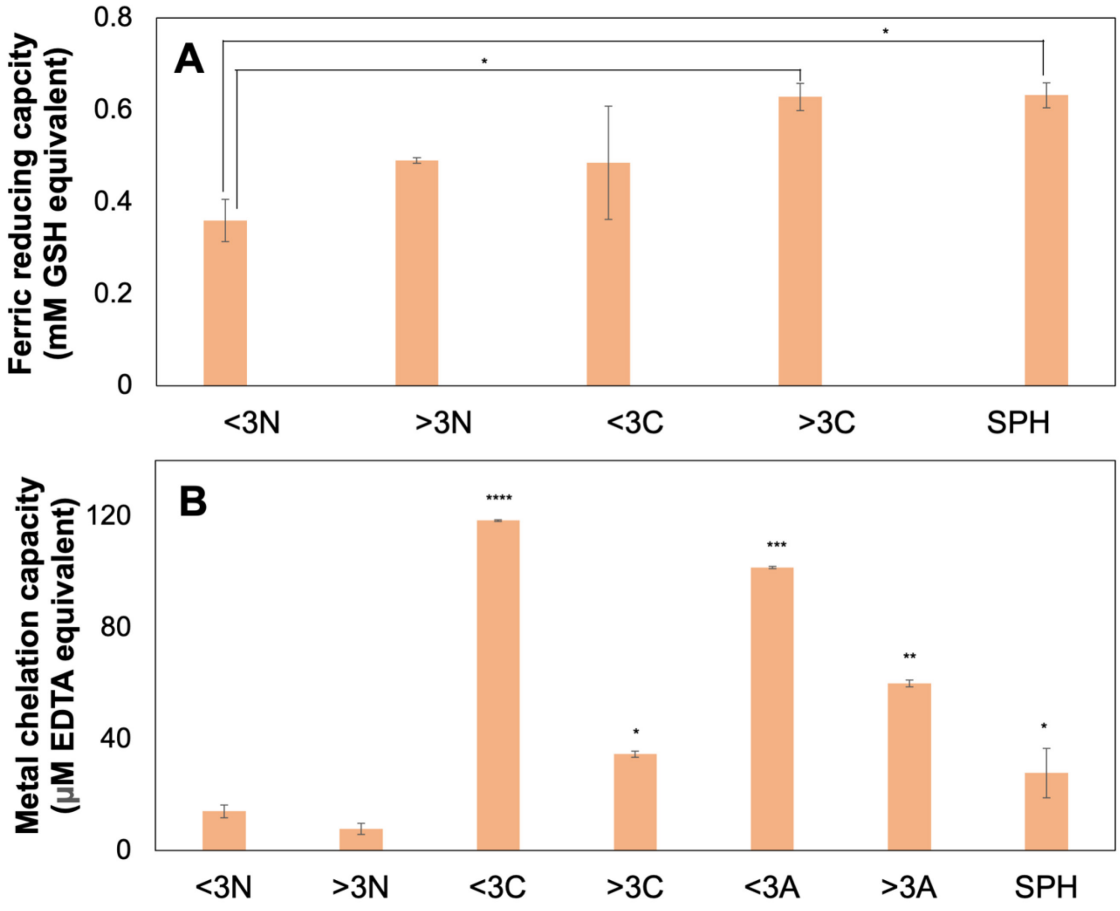

Figure 6.8: *Antioxidant capacity of SPH, permeate and retentate fractions: (A) ferric reducing capacity expressed as mM equivalent of GSH; (B) metal binding capacity expressed as µM equivalent of EDTA.*

Means denoted by asterix indicates a significant difference ($p < 0.05$) based on multiple comparison of means (Tukeys test) using one-way ANOVA.

The Fe (II) chelation assay determines the ability of analyte molecules to bind to pro-oxidant metal ions that can lead to the generation of free radicals through Fenton's reaction. Structure and amino acid composition of peptides play a major role in their ability to elicit metal chelation capacity.[245] Compared to SPH (28 ± 9 EDTA μM eqv.), more than 4-fold increase in metal chelation activity was observed for <3C (118 ± 0.4 EDTA μM eqv.), which had significantly higher metal chelation activity compared to other fractions. Higher abundance of histidine which was observed with <3C peptides, has been previously associated in the literature, with higher ability to chelate ferrous ions in previous studies.[76] Additionally, the anionic peptide fraction (<3A) was also found to exhibit high metal chelation activity (102 ± 0.4 EDTA μM eqv.) compared to SPH (Figure 6.8 B). The <3A fraction has high Glu+ Gln contents, Glu has been previously shown to improve Fe (II) chelation capacity.[246] Similarly, a 3-fold increase in ACE inhibitory activity was observed by Leeb *et al*., (2014) for EUF derived peptide fractions from dairy protein hydrolysate.[239] In contrast, fractionation of fish protein hydrolysate into anionic and cationic peptides derived from Rainbow trout via electrodialysis by Suwal *et al*., (2018) did not improve metal chelation capacity, although these fractions had enhanced radical scavenging activities.[247] Four-fold increase in activity is indication of the enrichment of bioactive peptides, improving the concentration and purity compared to SPH (Figure 6.8). Therefore, the fractionation using EUF from SPH could significantly improve its bioactivity and thus, the commercial value.

The unfractionated SPH was previously shown to have mineral contaminants in the range of ~10% of dry matter based on ash content (Table 2.1). These contaminants are also present in the ultrafiltered fractions as can be seen in the MW profile results (Section 6.3.2). Solid phase extraction with C-18 was therefore performed to reduce mineral contaminants and purify the peptides in the fractions. Ferric reducing capacity of the SPE-purified peptides was non-detectable.

It is not clear if acetonitrile-based elution from the C18 SPE impacted the composition or activity of these peptides. In contrast, the metal chelation activity remained consistent when compared to the original crude fractions (Figure 6.8). Both <3A and >3A fractions had significantly higher activity ($p<0.01$), the increase in activity is predicted to result from reduction of mineral contaminants (less than 0.5 kDa components as observed in <3A in Figure 6.5 and 6.6) in these samples. These results are indicative of the significance of fractionation of the SPH, which played a more significant role in bioactivity these assays compared to the mineral contaminants.

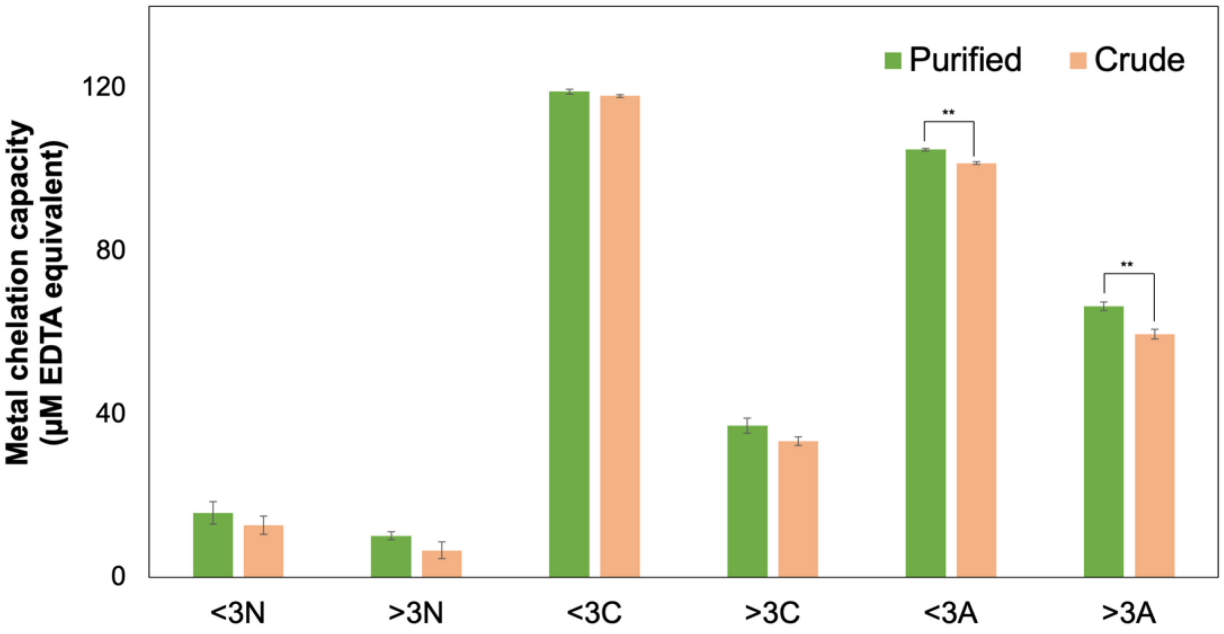

Figure 6.9: *Metal binding capacity of C-18 purified peptide fractions expressed as μM equivalent of EDTA.*

*significant difference observed at $p < 0.05$ with paired t test
**significant difference observed at $p < 0.01$ with paired t test

6.4. Conclusion and future outlook

The presented work describes a processing approach for improved selectivity for enrichment of salmon peptides using electro-ultrafiltration. Enrichment/diminution of basic/acidic amino acid residues in the peptide fractions based on the direction of voltage demonstrates the selectivity of this approach. Combined effects of pressure with the applied voltage facilitates the enrichment of peptides based on size and charge during processing that significantly impacts their functionality (metal chelation capacity). It is demonstrated in this study that EUF enrichment of SPH peptides resulted in higher acidic/basic amino acid residues in the peptide fractions based of direction of voltage. Separation significantly improved the antioxidant activities of both anionic (more than 3-fold) and cationic (4-fold) peptide fractions. In addition, this technique is selective, and environmentally friendly as it does not require application of solvent and consumes less energy compared to conventional chromatographic techniques. This strategy provides a viable approach for downstream separation of aqueous extracts with charged components. The present study also contributes to the understanding of peptide separation from a unique complex feed and the impact on functionality of the product results from selective enrichment of low molecular weight and charged peptides.

CHAPTER 7

Conclusion

Enriching peptides of desired properties is beneficial for developing ingredients or products with enhanced bioactivity. Development of these ingredients via the upcycling of fish by-products generated from processing is important for Canada and especially Atlantic Canada because of the abundance of these resources here, through a vibrant fisheries and aquaculture sector. Therefore, the present research utilized the fermentate derived through a unique and sustainable bioprocessing approach using lactic acid bacterial fermentation of Atlantic salmon by-products, as a natural source to further select functionally active peptides. Functional benefits of peptide bioproducts are dependent on their composition and physicochemical properties. In this light, development of sustainably derived peptide ingredients for their application in functional foods, animal feed, and nutraceuticals are of significance. Bioactive peptides have the potential to contribute towards mitigating disease states and promoting human health. For instance, food-based peptide product development efforts are currently directed at developing antioxidant, antimicrobial, antihypertensive, immunomodulatory, hypocholestrolaemic, hypolipidemic, and antithrombic activity. Peptide bioactivity is dependent on their conformation and ability to interact with various cellular components such as reactive oxygen species, enzymes, receptors and membranes. Therefore, the amino acid composition along with other physicochemical properties (polarity, size, charge etc.) and conformation primarily dictates the bioactive properties of peptides.

The fermentate generated via bacterial fermentation yields several distinct fractions. The protein hydrolysate fraction, in addition to being the most abundant in the fermentate, is also the most important for formulation of protein-based food/feed ingredients. This is due in part to its

high water solubility (suitable for use in health drinks; solubility also contributes towards higher bioavailability) and being readily available to be incorporated into different food matrices. In addition, because of its unique composition, the emulsion fraction was observed to have distinct features and functionality along with characteristics such as higher hydrophobicity that makes it amenable for unique applications in food substrates. The higher hydrophobicity and emulsifying ability can play a major role in incorporation into food matrices to reduce spoilage. Therefore, as described in Chapter 2, we were able to characterize the emulsion and aqueous protein hydrolysate fractions to identify distinct features associated with both fractions. Follow up studies can evaluate the impact of incorporating the emulsion as well as the other fractions into food matrices and the effect of functionality as a result of the same. The remainder of this thesis focused on the aqueous salmon protein hydrolysate fraction (SPH).

Stability and associated storage conditions for food and feed ingredients are important criteria to consider for downstream processing (for further fractionation/separation), storage, transportation of such products. The stability of salmon protein hydrolysate makes it suitable in its current form for further downstream processing and long-term storage. The absence of turbidity (indication of microbial contamination), consistent smell (flavor) and overall chemical stability during storage is predicted to be a reflection of its slightly acidic pH along with the lactic acid bacteria generated antimicrobial compounds (not evaluated here, though a subject of future work). The impact of processing approaches such as pasteurization would also need to be evaluated for food product development and application. Prior to incorporation into food or drinks, fish-based protein hydrolysate would require deodorization, bleaching and debittering, to reduce the effects of SPH incorporation on appearance and flavor of food products. The effects of each of these treatments will also need to be evaluated in subsequent studies.

Peptide composition plays a major role in determining biological activities even when generated from a given source using identical processing methods. Chemically distinct fractions of peptides can be isolated from a heterogenous mixture using simple, scalable separation strategies. It is evident that fractionation with electro-ultrafiltration is able to generate peptide fractions enriched in cationic or anionic peptides, depending on the polarity of the applied voltage. However, a drawback of the module used in the current study was the inability to carry out anode-directed filtration without causing damage to the electrodes. Although characterization of anode-directed permeate fraction was provided in this thesis, the impact of electrode dissolution on altering the separation process was not fully evaluated. One approach to consider in future developments with the EUF module should be the utilization of a more inert electrode material for the fabrication of the EUF module to prevent degradation of the electrode. Platinum or coated electrodes are resistant to electrolytic dissolution and reduces the complexation and redox reactions that can take place at the electrodes.

There is significant potential for considering the application of this separation technology in a wide variety of feed streams. For instance, in addition to cationic/anionic peptides, EUF has the potential to separate sulfated poly/oligosaccharides (negatively charged) from crude seaweed extracts to generate sulfate enriched poly/oligosaccharide fractions through anode-directed filtration to develop potent fraction with enhanced activity for applications in plant, animal and human health. The filtration module used in this study has an active membrane area of 20.6 cm^2 and is therefore only suitable for lab scale separations. Design and scale-up of electrode-assisted crossflow filtration module with higher active membrane area to increase permeate recovery require further considerations. For instance, the plate and frame configuration of the module used in this study has relatively lower surface area to volume ratio. Alternatively, spiral wound

membrane configuration maybe a more effective configuration for pilot and commercial separation.

Analytical techniques to evaluate and characterize peptide fractions plays a major role in ensuring consistent product characteristics and quality. Various chromatographic approaches such as size (GPC), polarity (RPLC), charge (SCX/WAX) provide a snapshot of peptide characteristics. Mass spectrometric analysis coupled to LC separation also enables the evaluation of the above-mentioned characteristics with certain limitations. For instance, conventional LC-MS/MS peptide sequencing approaches are limited by peptide size (~500-5000 Da) and charge (≥+2). We observed that sequencing and characterization of autolysis derived salmon peptides was significantly more challenging than tryptic peptides from the same sample. Cleavage specificity for enzymes such as mammalian trypsin and pepsin have been well characterized, and trypsin especially is conventionally used towards LC-MS/MS peptide sequencing. Whereas all commercial protease preparations and autolytic approaches lack cleavage specificity when used for generation of peptide fractions. Development of analysis tools suitable for peptidomic sequencing of fractions generated through processing approaches that lack cleavage specificity is essential for large scale evaluation of sequence data for peptide bioproduct development.

Salmon peptide product development needs the identification of peptides with desirable attributes and qualities to develop and commercialize peptide bioproducts. While a multitude of different bioactivities have been described in the literature associated with various physicochemical properties, evaluation of all of these aspects was not within the scope of this study. However, further evaluation of functional bioactive properties for peptide fractions such as antimicrobial activity, antihypertensive activity, antitumor activity can facilitate the development of a wide range of peptide bioproducts from different sources of protein hydrolysates. An improved

understanding of peptide physicochemical properties and their relationship to peptide functionality can boost research towards product development based on these relationships.

Future studies will aim to develop membrane processing approaches incorporating additional strategies. Outcome and learning from this study will be used to develop improved filtration modules with resistant electrode material and functionalized membrane surfaces along with the applied electric field, to enable the capacity to selectively separate components of a feed based on desired attributes such as charge, hydrophobicity, hydrophilicity, polarity and molecular weight. Another approach to be considered in future study is the use of membrane cascades, which has typically been used in the literature for the separation of binary/tertiary mixtures of proteins/oligosaccharides. Multidimensional selective fractionation of complex substrates is possible with the application of functionalized membrane cascades assisted by applied electric fields. In addition to the fractionation of peptides, these functionalized membrane-based filtration modules can also be assessed for their effectiveness in processing different complex feed material for separation of biomolecules with desired attributes. For instance, polysaccharide/oligosaccharide mixtures generated from the hydrolysis of seaweeds or microalgae (extracts) have several applications in food, health and agriculture (plant health). Fractions of seaweed extracts and oligosaccharides can then be evaluated for compositional differences in terms of mono-, and di-saccharide and polysaccharide content using HPLC, gel permeation chromatography and LC-MS/MS. Filtration modules will be used to measure membrane flux for different feed substrates, for separation of desired bioactives. Multiple filtration modules (with functionalized membranes) will be connected and configured with flexible tubing for development of the cascade system. The filtration systems described above will need to be assessed with model extracts prior to application with complex marine extracts. Optimization will need to be carried

out to determine the flow rate at each stage of cascading based on the profiles of flux, fractionation and fouling over time at each of the membrane modules.

Commercial production of this salmon by-product protein hydrolysate and enriched fractions can thus, open further possibilities towards development of unique peptide bioproducts that can have beneficial health outcomes for humans, animals and pets. Effectively utilizing these waste streams for functional food/feed development or in improving food storage prevents competition for existing resources for developing these ingredients. Further research will be required to elucidate nutritional, textural, and sensory changes associated with salmon by-product preparation and incorporation into food matrices. Additionally, evaluation of *in vivo* activity is necessary to determine functionality in target biological systems while being safe for consumption and beneficial towards as preventative health practices. various innovations and strategies in membrane processing that have the scope for improving selectivity and separation efficiency, while reducing the impact of fouling.

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APPENDIX

Table of Contents

Table A1: *List of top 20 most abundant proteins (based on peptide spectral matches) from the salmon protein hydrolysate as determined by bottom-up proteomics (LC-MS/MS)*

Source protein
myosin heavy chain, fast skeletal muscle-like isoform X1 OS=Salmo salar OX=8030 GN=LOC106564162 PE=3 SV=1
Calcium-transporting ATPase OS=Salmo salar OX=8030 GN=LOC106579065 PE=3 SV=1
aldehyde dehydrogenase family 7 member A1 homolog OS=Salmo salar OX=8030 GN=AL7A1 PE=2 SV=1
acidic mammalian chitinase-like OS=Salmo salar OX=8030 GN=LOC106565087 PE=3 SV=1
Actin alpha 1-1 OS=Salmo salar OX=8030 GN=LOC106564730 PE=2 SV=1
Beta-hexosaminidase OS=Salmo salar OX=8030 GN=hexb PE=3 SV=1
mucin-5AC-like OS=Salmo salar OX=8030 GN=LOC106595510 PE=4 SV=1
Carboxypeptidase A1 precursor OS=Salmo salar OX=8030 GN=CBPA1 PE=2 SV=1
Alpha-1,4 glucan phosphorylase OS=Salmo salar OX=8030 GN=pygma PE=2 SV=1
Ferritin OS=Salmo salar OX=8030 GN=FRIM PE=2 SV=1
eosinophil peroxidase OS=Salmo salar OX=8030 GN=LOC100380666 PE=4 SV=1
vitellogenin OS=Salmo salar OX=8030 GN=LOC100136426 PE=4 SV=1
Glyceraldehyde-3-phosphate dehydrogenase OS=Salmo salar OX=8030 PE=2 SV=1
Ferritin, heavy subunit OS=Salmo salar OX=8030 PE=2 SV=1
Alpha-galactosidase OS=Salmo salar OX=8030 GN=NAGAB PE=2 SV=1
fibrillin-2-like OS=Salmo salar OX=8030 GN=LOC106613712 PE=4 SV=1
Fructose-bisphosphate aldolase OS=Salmo salar OX=8030 GN=LOC100194624 PE=3 SV=1
alpha-actinin-3-like OS=Salmo salar OX=8030 GN=LOC106577856 PE=4 SV=1
mucin-5AC-like OS=Salmo salar OX=8030 GN=LOC106593959 PE=4 SV=1
cathepsin L1-like OS=Salmo salar OX=8030 GN=LOC106560797 PE=3 SV=1

Table A2: *List of top 20 most abundant proteins (based on peptide spectral matches) identified from the salmon emulsion as determined by bottom-up proteomics (LC-MS/MS).*

Source protein
titin-like isoform X1 OS=Salmo salar OX=8030 GN=LOC106586456 PE=4 SV=1
myosin heavy chain, fast skeletal muscle-like isoform X1 OS=Salmo salar OX=8030 GN=LOC106564162 PE=3 SV=1
Calcium-transporting ATPase OS=Salmo salar OX=8030 GN=LOC106579065 PE=3 SV=1
vitellogenin OS=Salmo salar OX=8030 GN=LOC100136426 PE=4 SV=1
fibrillin-2-like OS=Salmo salar OX=8030 GN=LOC106573567 PE=4 SV=1
collagen alpha-1(XII) chain isoform X1 OS=Salmo salar OX=8030 GN=col12a1 PE=4 SV=1
nebulin OS=Salmo salar OX=8030 GN=neb PE=4 SV=1
Fructose-bisphosphate aldolase OS=Salmo salar OX=8030 GN=LOC100194624 PE=3 SV=1
Alpha-1,4 glucan phosphorylase OS=Salmo salar OX=8030 GN=pygma PE=2 SV=1
Actin alpha 1-1 OS=Salmo salar OX=8030 GN=LOC106564730 PE=2 SV=1
Glyceraldehyde-3-phosphate dehydrogenase OS=Salmo salar OX=8030 GN=G3P PE=2 SV=1
alpha-actinin-3-like isoform X1 OS=Salmo salar OX=8030 GN=LOC106608893 PE=4 SV=1
myosin-binding protein C, slow-type isoform X1 OS=Salmo salar OX=8030 GN=mybpc1 PE=4 SV=1
Myosin regulatory light chain 2-2 OS=Salmo salar OX=8030 GN=mlc-2 PE=2 SV=1
enolase 3-1 OS=Salmo salar OX=8030 GN=LOC100196671 PE=3 SV=1
Creatine kinase-1 OS=Salmo salar OX=8030 GN=ckm1 PE=2 SV=1
collagen alpha-3(VI) chain-like isoform X5 OS=Salmo salar OX=8030 GN=LOC106581666 PE=4 SV=1
keratin, type I cytoskeletal 13-like OS=Salmo salar OX=8030 GN=LOC106564958 PE=3 SV=1
Pyruvate kinase OS=Salmo salar OX=8030 GN=pk PE=2 SV=1
filamin-C-like isoform X3 OS=Salmo salar OX=8030 GN=LOC106561692 PE=4 SV=1

Illustrations on set-up of filtration assembly in steps

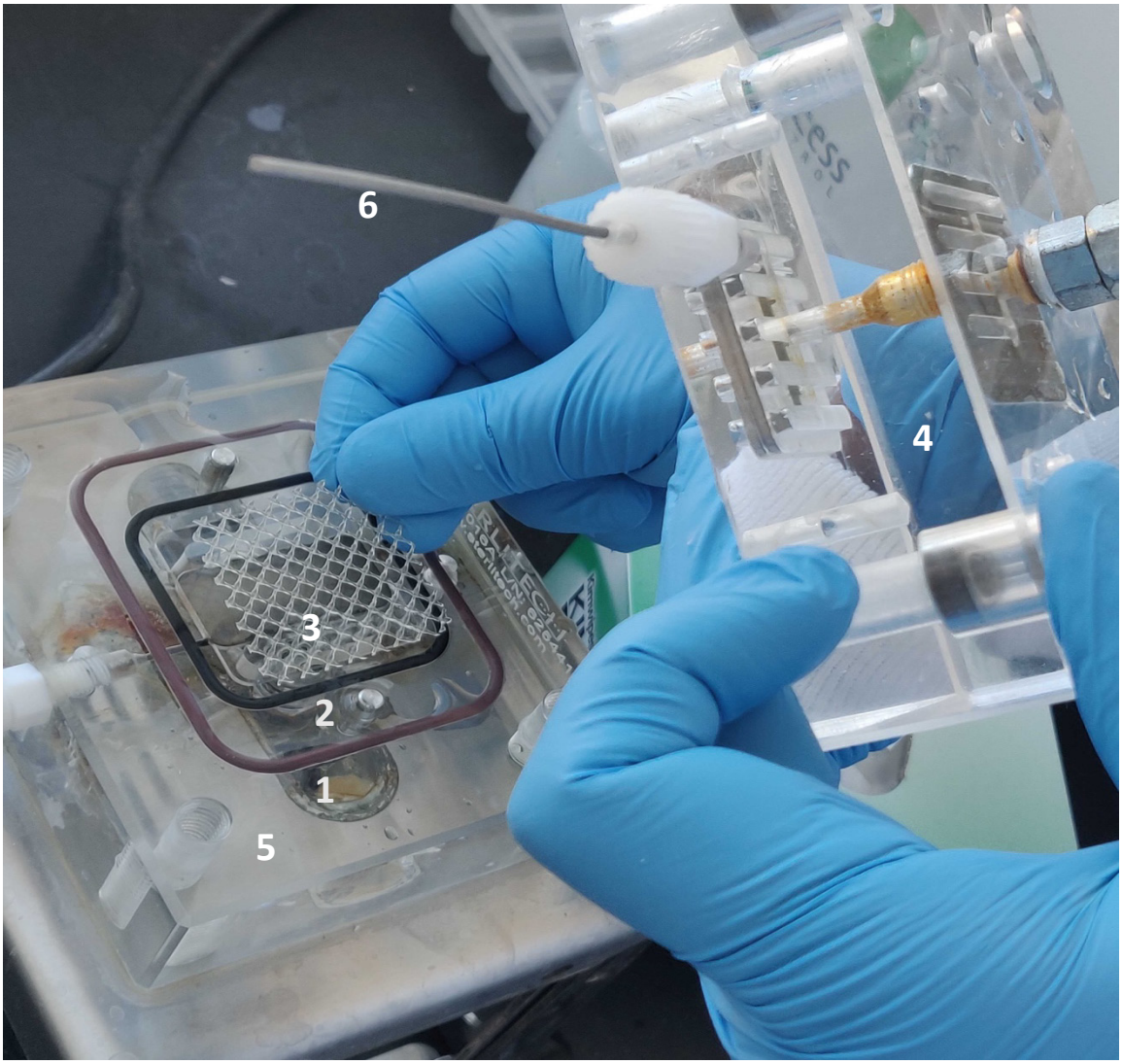

Figure A1. *Photograph of set up of filtration module – placement of feed spacer. (1) Outer O-ring, (2) Inner O-ring, (3) Spacer, (4) Top plate, (5) Bottom plate, (6) Electrode on permeate side*

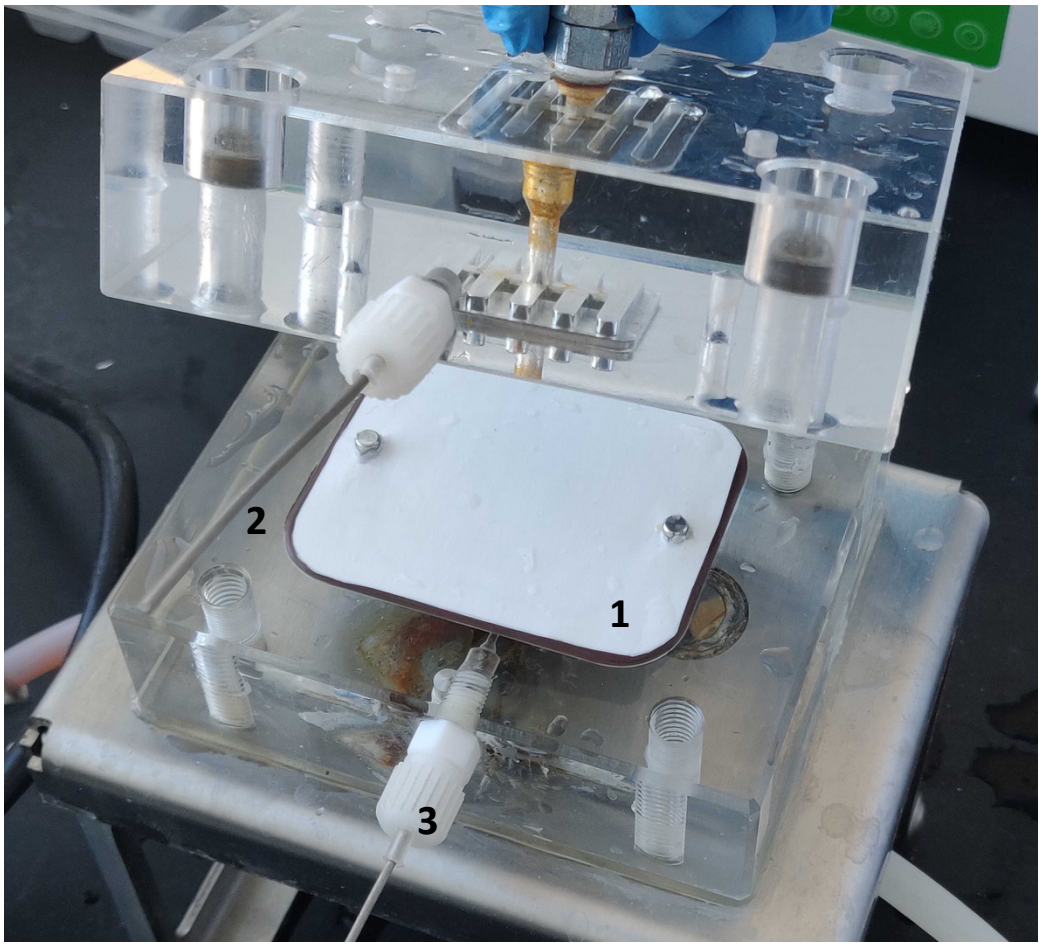

Figure A2. *Photograph of set up of filtration module – placement of flat sheet membrane. (1) Membrane, (2) Electrode on permeate side, (3) Electrode on feed side*

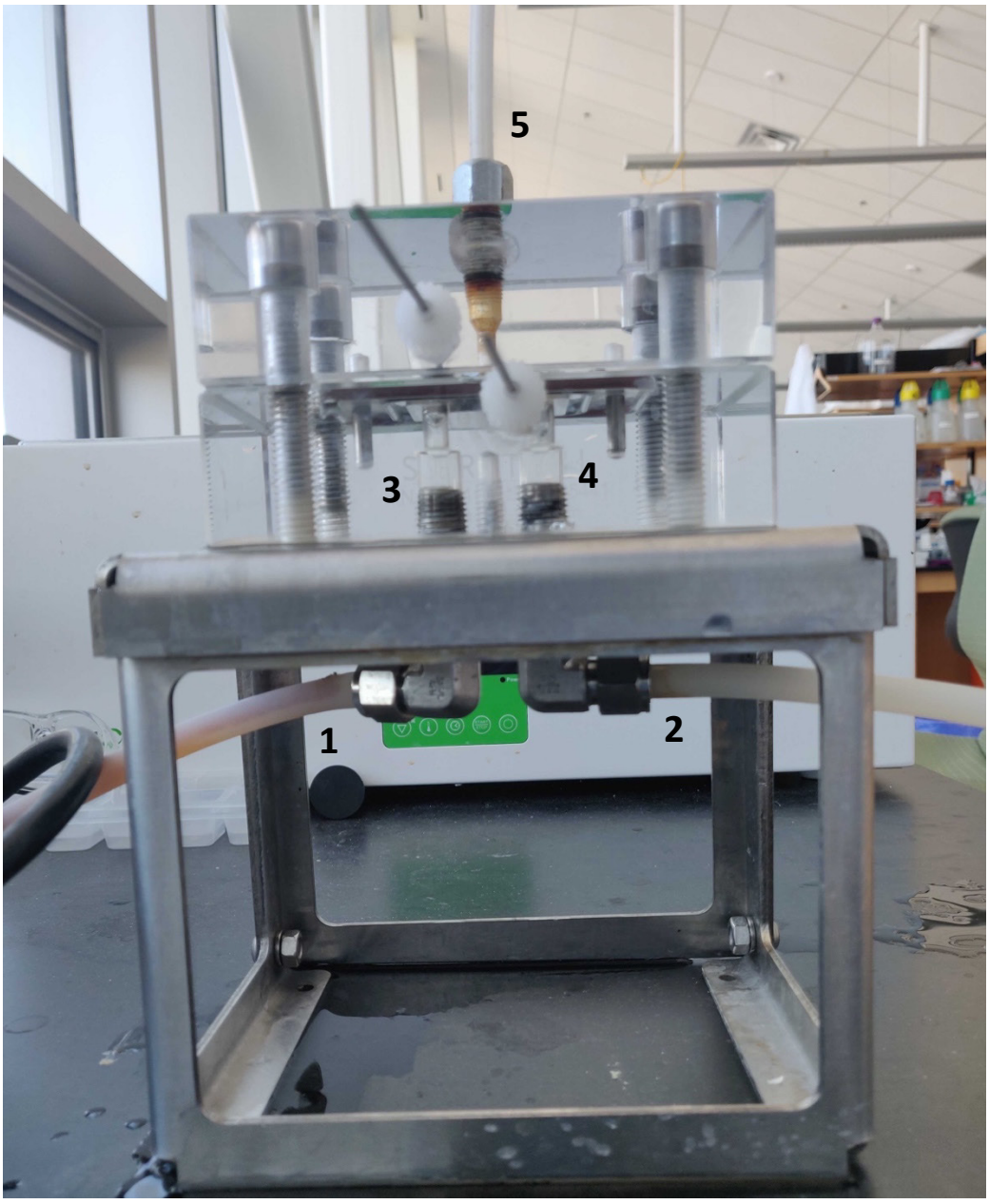

Figure A3. *Photograph of set up of filtration module – fastened module with membrane. (1) Feed line, (2) Retentate line, (3) Feed channel inside the module, (4) Retentate channel inside the module, (5) Permeate line*

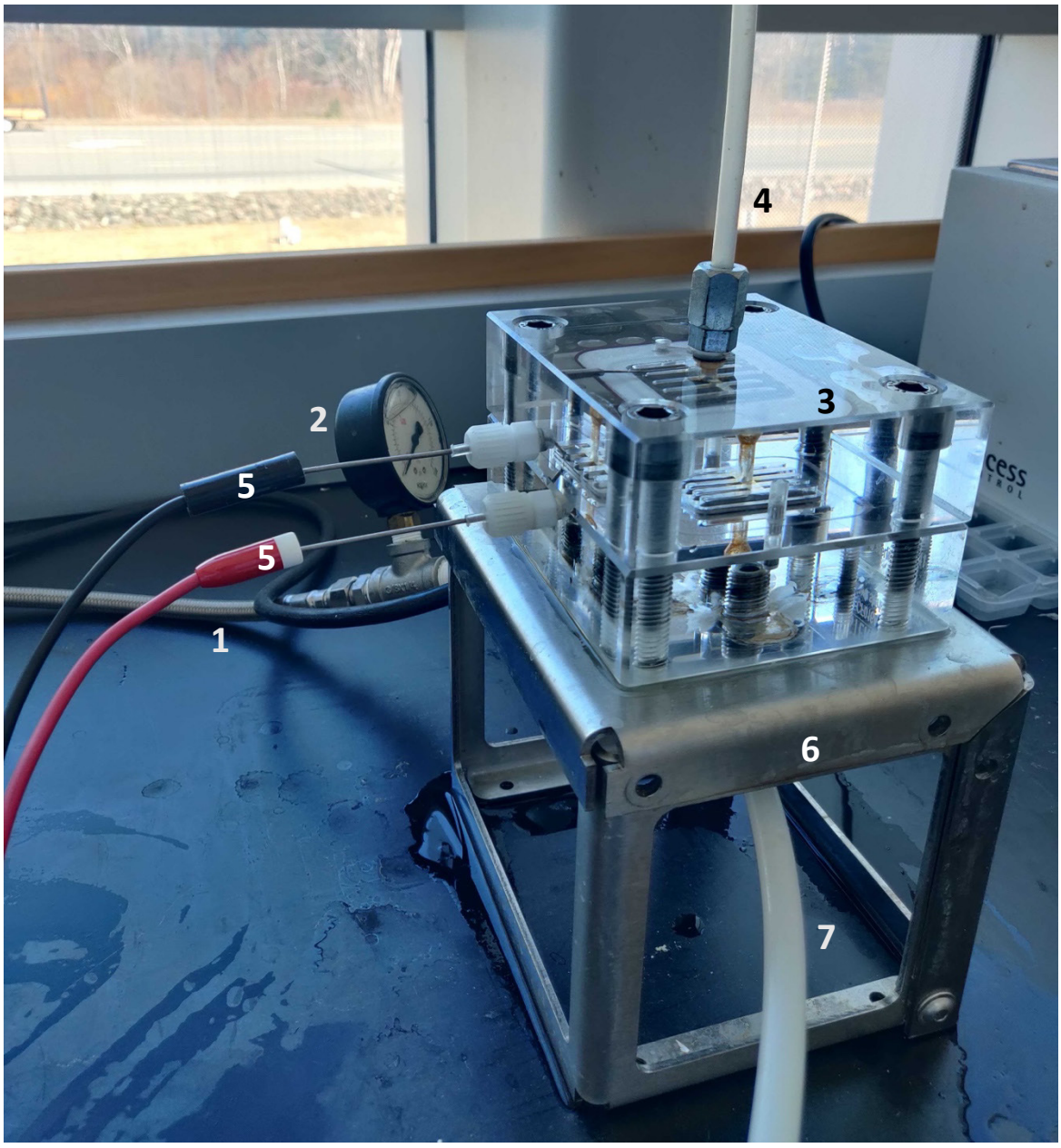

Figure A4. *Photograph of set up of filtration module – complete set up with electrodes connected. (1) Feed line, (2) Pressure gauge, (3) Module with flat sheet membrane, (4) Permeate line, (5) Electrodes connected to power supply, (6) Stand for the module, (7) Retentate line*

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