PROTEIN ISOLATES AND HYDROLYSATES FROM ATLANTIC SEA CUCUMBER (*Cucumaria frondosa)*: CHARACTERIZATION AND BIOACTIVITIES

By

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ABSTRACT

North Atlantic sea cucumber (*Cucumaria frondosa*) or orange-footed sea cucumber is a benthic marine echinoderm found in Northwest Atlantic waters. It is harvested mainly for its edible body wall and muscle bands. The aquaphyrangeal bulb or flower is considered as a secondary marketable product and internal organs including gonads, respiratory tracts and intestine are discarded as processing by-products, which generate a high volume of biological waste. However, all the body parts of the sea cucumber are rich in protein. The objective of this research was to recover the proteins from sea cucumber tissues and produce protein hydrolysates with bioactive properties associated with antioxidative and angiotensin-I-converting enzyme (ACE) inhibitory activities following a multidisciplinary approach. Proteins were isolated from the body wall, flower and internal organs of *Cucumaria frondosa* using a pH-shift method. Isolated proteins were evaluated for their physicochemical properties including solubility, emulsifying and foaming properties, water- and oil-holding capacities, surface hydrophobicity, content of sulfhydryl and disulfide groups along with FTIR analysis for identifying the functional groups of proteins and amino acid compositional analysis. Findings of this research revealed that sea cucumber-derived protein isolates could be used as a natural alternative to soy protein isolates as well as a source of balanced dietary proteins. Lyophilized protein isolates were then hydrolysed using food grade commercial enzymes, namely Alcalase, Corolase and Flavourzyme individually and in combination. The protein hydrolysates so prepared were evaluated for their antioxidant potential in different systems including radical scavenging assays such as the 2-2-diphenyl-1-picrylhydrazyl (DPPH) radical, the2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation, and hydroxyl radical

scavenging assays. In addition, metal chelating, the reducing power, and β-carotene bleaching assays in oil-in-water emulsion as well as thiobarbituric acid (TBA) assays in meat-model system were performed. Furthermore, hydroxyl and peroxyl radical induced DNA strand scission inhibitory activity assay and the cupric ion- induced human low-density lipoprotein (LDL) peroxidation inhibitory activity assays were performed. Finally, physicochemical properties of protein hydrolysates were evaluated based on their solubility, color and water holding capacity in a meat model system. The samples treated with a combination of Alcalase and Flavourzyme exhibited strong antioxidant potential and better functional properties compared to that of other treatments as well as their untreated counterparts of all tested sea cucumber body parts. The selected protein hydrolysates were also assessed for their ACE inhibitory activity to examine the potential multifunctional behaviour. All these findings along with the amino acid profiles demonstrated that the samples hydrolyzed with Alcalase and Flavourzyme exhibited more favourable properties. Results demonstrated that sea cucumber protein hydrolysates have great potential for use as nutritional supplements. Protein hydrolysates with highest antioxidant activity were further analysed for their molecular weight distribution and amino acid sequence using liquid chromatography- mass spectrometry (LC-MS/MS). The identified peptide sequences were then subjected to *in silico* analysis employing biotechnological tools PepRank, BIOPEP-UWM data base, ToxinPred and SwissADME. Virtual screening of bioactive peptides predicted that sea cucumber-derived peptides have the potential to develop as therapeutics in oral administration. Thus, the findings of the current study revealed the potential applications of sea cucumber-derived protein isolates, hydrolysates and bioactive peptides in functional food, nutraceutical, dietary supplement as well as natural therapeutics.

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A	Alcalase
AA	Amino acid analysis
AAA	Aromatic amino acids
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS	2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulfonate
ACE	Angiotensin-I-converting enzyme
ADME	Absorption, distribution, metabolism and excretion
AGC	Automatic gain control
ANOVA	Analysis of variance
AT-I	Angiotensin I
AT-II	Angiotensin II
ATR	Attenuated total reflectance
BCA	Bicinchoninic acid
BCAA	Branched-chain amino acids
BHT	Butylated hydroxytoluene
BPB	Bromophenol blue
BW	Body wall
BW (A+F)	Body wall treated with Alcalase and Flavourzyme
BW (C+F)	Body wall treated with Corolase and Flavourzyme
BWA	Body wall treated with Alcalase
BWC	Body wall treated with Corolase

BWF	Body wall treated with Flavourzyme
BWI	Body wall protein isolates
C	Corolase
CE	Capillary electrophoresis
CIE	International commission of illumination
DFO	Department of Fisheries and Oceans
DH	Degree of Hydrolysis
DHA	Docosahexaenoic acid
DMPO	5,5-Dimethyl-1-pyrroline-N-oxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EAA	Essential amino acids
EAI	Emulsification activity index
EDTA	Ethylenediaminetetraacetic acid
EDUF	Electrodialysis-ultrafiltration
EPR	Electron paramagnetic resonance
ESI	Emulsification stability index
ET	Electron transfer
F	Flavourzyme
FAO	Food and Agriculture Organization
FCI	Foaming capacity index
FL	Flower

FL (A+F)	Flower treated with Alcalase and Flavourzyme
FL (C+F)	Flower treated with Corolase and Flavourzyme
FLA	Flower treated with Alcalase
FLC	Flower treated with Corolase
FLF	Flower treated with Flavourzyme
FLI	Flower protein isolates
FRAP	Ferric reducing antioxidant power
FSI	Foaming stability index
FTIR	Fourier-transform infrared spectroscopy
GC	Gas chromatography
HAT	Hydrogen atom transfer
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donors
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHL	Hippuryl-L-histidyl-L-leucine
HPLC-MS/MS	High-performance liquid chromatography -mass spectrometry/ mass spectrometry
IC50	Half maximal inhibitory concentration
IN	Internal organs
IN (A+F)	Internal organs with Alcalase and Flavourzyme
IN (C+F)	Internal organs with Corolase and Flavourzyme
INA	Internal organs treated with Alcalase

INC	Internal organs with Corolase
INF	Internal organs with Flavourzyme
INI	Internal organs protein isolate
LC-MS/MS	Liquid chromatography- mass spectrometry / mass spectrometry
LDL	Low-density lipoprotein
MALDI	Matrix-assisted laser desorption ionisation
MDA	Malondialdehyde
MS	Mass spectroscopy
MUFA	Monounsaturated fatty acids
NEAA	Non-essential amino acids
NO	Nitric oxide
OPA	*O*-phthaldialdehyde
ORAC	Oxygen radical absorbance capacity
pI	Isoelectric point
PUFA	Polyunsaturated fatty acids
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
ROTB	Rotatable bonds
RP-HPLC	Reversed-phase-high performance liquid chromatography
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SET	Single electron transfer

SFA	Saturated fatty acids
SH	Sulfhydryl group
SPI	Soy protein isolates
SS	Disulfide bond
STPP	Sodium tripolyphosphate
SVM	Support vector machine
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
TNBS	Trinitrobenzenesulfonic acid
TOF	Time of flight
TPSA	Topological polar surface area
UHPLC	Ultra-high performance liquid chromatography
WHO	World Health Organization

CHAPTER 1

INTRODUCTION AND OVERVIEW

Sea cucumbers are marine invertebrates belonging to the class Holothuroidea and phylum Echinodermata (Zhong, Khan and Shahidi, 2007; Hossain, Dave and Shahidi, 2020). The impressive profile of nutrients and plethora of bioactive compounds enhance high commercial value to sea cucumber (Senadheera, Dave and Shahidi, 2020). For decades, sea cucumber has been viewed as a seafood delicacy in East Asia and used in folk medicine (Bordbar, Anwar and Saari, 2011). A multitude of bioactive compounds derived from sea cucumber exert unique biological and pharmacological activities such as antioxidant, anticancer, anti-inflammatory, antimicrobial, anticoagulant/ antithrombotic, and wound healing (Shi et al., 2016). The most abundant chemical component of sea cucumber, on a dry weight basis, is protein, which accounts for 40- 60% of its total composition (Xu, Zhang and Wen, 2018). Among more than 1500 identified species, *Cucumaria frondosa* (orange footed sea cucumber) is known as the most abundant sea cucumber species found in the North Atlantic Ocean (Hossain et al., 2020). The body wall of sea cucumber is the major marketable portion, which is usually processed into a dried product. However, *Cucumaria frondosa* has a thin body wall and it is relatively smaller in size compared to other sea cucumber species in the global market (Lin et al., 2018). Thus, this species accounts for low economic value and remained underutilized compared to the other commercial species (Zhang, He, Bonneil and Simpson, 2020). In addition to the body wall, some of the sea cucumber processing industries also sell the tentacles (flower) in dried form (Hossain et al., 2020). All the other remaining tissues, including internal organs are

discarded as processing waste, a fraction that represents up to 50% of the total body mass of the animal (Mamelona, Saint-Louis and Pelletier, 2010).

In recent years, much attention has been paid to upgrading seafood processing waste to value-added by-products (Shahidi and Ambigaipalan, 2015; Liu, Ramakrishnan and Dave, 2020). In general, marine food processing waste has been recognized as a rich source of protein (Zhang et al., 2020). There has been a growing research interest to recover proteins from the waste and develop high value marketable products. In this regard, protein isolation and production of protein hydrolysates are considered effective biorefinery methods (Kristinsson and Liang, 2006; Liu et al., 2020). Protein isolation using pH-shift method and employing enzyme hydrolysis are the most popular strategies of upgrading food proteins (Girgih, Udenigwe, Hasan, Gill and Aluko, 2013; Ambigaipalan, Al-Khalifa and Shahidi, 2015). The most frequently used enzymes are derived from plants, animals and microbial sources (Kristinsson and Rasco, 2000; Rodríguez-Vázquez et al., 2020). However, microbial proteases, such as Alcalase, Flavourzyme, and Corolase are popular in industrial production due to their favourable operational conditions (Kristinsson and Rasco, 2000).

Investigation of intrinsic physicochemical and functional characteristics of recovered protein in food and biological model systems has diversified their use in the nutraceutical and functional food industry (Du et al., 2020). More recently, bioactive peptides produced from enzymatic hydrolysis of food protein have been in the spotlight for their potential pharmaceutical and therapeutic applications (Udenigwe and Aluko, 2012). Several studies conducted on peptides derived from sea cucumber protein hydrolysates have demonstrated their potential biological activities, including antioxidant, angiotensin

converting enzyme (ACE) inhibition, and antiproliferative effects (Tripoteau, Bedoux, Gagnon and Bourgougnon, 2015). Thus far, most of the studies in protein hydrolysates and bioactive peptides have been conducted focusing on *in vitro* activity measurements combined with purification and sequencing methods which belong to conventional approaches (Zhang et al., 2020). Currently, bioinformatic tools are being developed and utilized in *in silico* analysis, which is recognized as an effective alternative method for predicting and discovering potent peptides derived from food proteins (Udenigwe, 2014).

Numerous studies have been conducted on the preparation of isolates and hydrolysates from different protein sources, including marine organisms and their by-products, such as capelin (Shahidi, Han and Synowiecki, 1995; Amarowicz and Shahidi, 1997), shrimp shells (Ambigaipalan and Shahidi, 2017), sardine, mackerel, bogue and small-spotted catshark (García-Moreno et al., 2014), blue-spotted stingray (Wong et al., 2019), and Atlantic salmon (Liu et al., 2020), among others. In contrast, a very limited number of studies have reported the bioactive potential of Atlantic sea cucumber by-products, including antiviral activities of flower and internal organs (Tripoteau et al., 2015), antioxidant activities (Mamelona et al., 2010a) and functional properties (Yan, Tao and Qin, 2016) of viscera. Few of the recent studies have focused on the hydrolysis of the whole animal to examine the potential of using hydrolysates as antiaging agents and identification of antioxidative peptides (Lin et al., 2018; Zhang et al., 2020). To the best of our knowledge, protein recovery and enzymatic hydrolysates of different tissues of *Cucumaria frondosa* have not been comparatively studied to evaluate their physicochemical properties and assess their antioxidant and ACE inhibitory potentials. Moreover, there is no report available on determining the effect of sequential use and individual endopeptidases and

exopeptidases on the production of protein hydrolysates from Atlantic sea cucumber discards.

Thus, the main objective of this study was to explore the functional, physicochemical and bioactive potential of different body parts of the Atlantic sea cucumber *Cucumaria frondosa* for protein isolates and hydrolysates and to predict the potent peptide sequences using bioinformatics integrated approach.

1.1.Objectives of the present study

The specific objectives of the study are summarised below.

1) Investigation of physiochemical properties of protein isolates prepared from body wall, flower and internal organs of the Atlantic sea cucumber *Cucumaria frondosa.*
2) Determination of the effect of sequential and individual use of selected endopeptidases and exopeptidases in the production of protein hydrolysates from Atlantic sea cucumber protein isolates.
3) Evaluation of the antioxidant efficacy of protein hydrolysates in food and biological model systems and comparative analysis of ACE inhibitory activity of identified potent hydrolysates.
4) Analysis of the molecular weight distribution of peptides produced during the enzyme hydrolysis and *in silico* simulation and predictions of bioactive potentials and physicochemical properties of sea cucumber derived peptides using bioinformatic tools.

CHAPTER 2

LITERATURE REVIEW

Sea cucumbers are phylogenetically related to sea urchins, sea stars and sea lilies. (Zhong et al., 2007; Bordbar et al., 2011). As one of the highly diverse groups of marine invertebrates, sea cucumbers are found in most of the benthic marine environments as well as the deep seas and oceans across the world (Bordbar et al., 2011). Their habitats vary from hard substrates like rocks, coral reefs to soft aquatic plants and some species show epibiosis relationship with other invertebrates (Purcell et al., 2013). Sea cucumbers are performing important roles in the marine ecosystem by being involved in maintaining water quality and nutrient recycling by feeding on detritus (Sun, Hamel, Gianasi, Graham and Mercier, 2020).

2.1 Sea cucumber: Classification

The class Holothuroidea is categorized into three subclasses: *Dendrochirotacea, Aspidochirotacea* and *Apodacea*. These subclasses are further divided into six orders, namely *Aspidochirotida, Apodida, Dactylochirotida, Dendrochirotida, Elasipodida* and *Molpadida* (Conand 2006). The basis for differentiation of the orders depends on factors including presence or absence of the podia, oral retractor muscles, respiratory trees and curverian tubules and shape of the oral tentacles (Kamarudin et al., 2010). Habitat and behavior of the sea cucumbers are also vital factors for categorizing them into orders (Conand 2006). To date, taxonomic levels of sea cucumber are poorly understood due to lack of proper characterization of intraspecies variations.

2.2 Sea Cucumber: Biology

These marine invertebrates possess an elongated gelatinous soft body that resembles a cucumber (Zhong, et al., 2007; Bordbar et al., 2011). Pentamerous radial symmetry can be observed due to their five longitudinal rows of tube feet known as podia. Three rows are located on the ventral side of the body and two are on the dorsal side (Figure 2.1). The dorsal podia are known as locomotory podia or papillae. (Zamora, Yuan, Carton and Slater, 2018). Sea cucumbers use these locomotory podia or tube feet to move along the ocean floor.

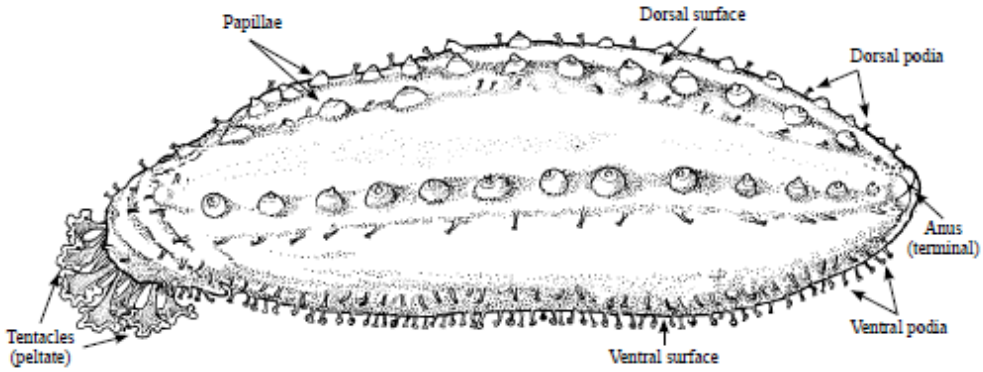


Figure 2.1 External anatomy of sea cucumber (Source: Purcell, Samyn and Conand, 2012)

Around 1500 sea cucumbers species have been discovered across the world and most of the recent studies stated that the number of unexplored sea cucumber species is still higher than the described species (Hossain et al., 2020). However, the majority of the existing commercial species belong to *Aspidochirota* and few harvestable species come under *Dendrochirota*. The genus *Cucumaria* from the family *Cucumariidae* is one of the important commercial species that belongs to the order *Dendrochirota* (Conand 2006; Zamora et al., 2018)

Sea cucumber *Cucumaria frondosa* (orange footed sea cucumber) has been the focus of commercial fisheries in the North Atlantic region (Hamel and Mercier, 2008; Sun 2020). The orange footed sea cucumber has the ability to grow to a maximum length of 40-50 cm and a weight of 100-500 g (Hossain et al., 2020). *Cucumaria frondosa* is identified as a column feeder who feeds on suspended materials including phytoplankton, zooplankton and organic matter (Sun et al., 2020). The growth and spatial distribution of *Cucumaria frondosa* depend on environmental conditions like temperature, salinity, turbulence, food supply. The degree of disturbance has a major impact on their growth. Moreover, the uniform and seasonal pattern growth of sea cucumbers in shallow and deeper water was reported by Sun et al. (2020). There was a remarkable development of the growth of sea cucumbers during periods of spring and summer with stable environment conditions (Mercier and Hamel, 1998). Mainly, seasonal changes may have a direct influence on the growth of species as changes in phytoplankton concentration, water temperature, light intensity like environmental factors are proposed to be major influencing cues involved with spawning in sea cucumber (Hamel et al., 2019).

2.3 Canadian sea cucumber fishery

With the recent expansion of marine invertebrate fisheries across the world, sea cucumber has received much attention as one of the high valued commodities both in the food and biomedical industries (Zhong et al., 2007). The yield of invertebrate fisheries is reported to be low compared to the finfish industry. However, the commercial harvesting of invertebrates is widely spread due to their high economic value. Commercial exploitation

of sea cucumber started in 1970s in North America (Hamel and Mercier, 1996). The earliest sea cucumber fisheries reported in Canada initiated during late 1980s and emerged as an industry in the early 1990s with the opening of the Asian market (Hamel and Mercier, 1998). The first official landing of commercial sea cucumber in Canada was in 1980 in British Columbia (Hamel and Mercier, 2008).

Moreover, world holothurian fisheries have been categorized according to the geographical area, namely tropical fisheries and temperate fisheries. Temperate fisheries are confined to the North Pacific region and include Western and Eastern Pacific regions (Hamel and Mercier, 2008). North Western USA and British Colombia in Canada come under Eastern Pacific Ocean region and are famous for red sea cucumber fisheries (*Parastichopus californicus)* while Eastern Coast of Canada is categorized under Atlantic region and famous for the harvesting of orange footed sea cucumber (*Cucumaria frondosa*) (Hamel and Mercier, 2008; Purcell et al., 2013).

2.3.1 Atlantic region

As one of the abundant holothurian species, *Cucumaria frondosa* is widely distributed throughout the east coast of Canada and in numerous other locations around the North Atlantic Ocean ranging from northern Europe and Scandinavia to the Faeroe Islands and Iceland in the eastern and western Atlantic regions (Hamel and Mercier 1996). *Cucumaria frondosa* densities are present in Atlantic provinces of Canada including Nova Scotia, New Brunswick and Newfoundland (So, Hamel and Mercier, 2010). Furthermore, the orange footed sea cucumber prefers rocky or sandy substrates and abundant in offshore than inshore.

2.3.2 Pacific region

The west coast of Canada is famous for the harvesting of giant red sea cucumber (*Parastichopus californicus*), and the first official landing was reported in 1980 in British Columbia (Hamel and Mercier, 2008). According to the data published by the Department of Fisheries and Oceans (DFO, 2002) of Canada, sea cucumber fishery in British Columbia which is categorized into four geographical areas including the east, west, central and north coast of the Vancouver Island; both central and north coast account for approximately 80% of the fishery (Hamel and Mercier, 2008).

2.4 Chemical composition and nutritional profile

Sea cucumbers are rich in nutrients and considered as one of the significant seafood with curative properties (Zhong et al., 2007; Bordbar et al., 2011; Hossain et al., 2020). Globally, sea cucumbers are exploited mainly from wild fisheries and considered as luxury seafood items, mainly targeting the Asian market (Xu et al., 2018)

Most of the Asian countries process sea cucumber into a dried product known as "beche-de-mer" or "trepang" and rank them according to their commercial value (Wen, Hu and Fan, 2010; Khotimchenko, 2018). The commercial value is often determined by the species, abundance, appearance, odor, color, thickness of the body wall and market demand (Wen et al., 2010). From a nutritional viewpoint, sea cucumber possesses an impressive nutritional profile enriched with vitamins like thiamine, riboflavin, and niacin as well as minerals such as magnesium, calcium, zinc and iron (Zhong et al., 2007; Bordbar et al., 2011). However, composition and bioactivity of sea cucumbers varies among species and is based on seasonal variations and feeding pattern (Olivera-Castillo et al., 2018).

Moreover, further processing steps hydration and rehydration might have an impact on overall proximate composition by causing loss of water and minerals and thereby increasing the protein and lipid content in processed sea cucumbers (Zhong et al., 2007). According to the proximate composition of most of the sea cucumbers, moisture content falls within the range of 82.0 – 92.6% and the most abundant nutrient is the protein which varies from 2.5 to 13.8%, depending on the species and other environmental factors (Zhong et al., 2007; Bordbar et al., 2011). It contains a lower level of fat than most other foods and with ash and carbohydrate contents of around 1.5 - 4.3% and 0.2 - 2.0%, respectively (Bordbar et al., 2011; Xu et al., 2018).

When considering the protein present in the body wall of sea cucumber, the major part has been reported to consist of insoluble collagen, however, this was not found to be the case for the species tested from waters of Newfoundland (Hossain et al., 2020). It has been reported that the unique characteristics of sea cucumber correlate with the composition of its essential amino acids and richness in lysine, arginine, and tryptophan (Zhong et al., 2007). Moreover, studies of eight commercial sea cucumbers by Wen et al. (2010) revealed that glycine, glutamic acid, aspartic acid, alanine and arginine were the most prominent amino acids with predominance of glycine. Furthermore, the presence of threonine, tyrosine and phenylalanine resulted in the low lysine/arginine ratio that may affect the concentration of cholesterol in the serum and aorta (Wen et al., 2010). In studies conducted by Zhong et al. (2007) based on *Cucumaria frondosa*, glutamic acid was the dominant amino acid among all seventeen amino acids detected. These authors also reported a higher ratio of essential to non-essential amino acids in the samples with internal organs compared to the samples without internal organs.

Fatty acid profiles of the same cucumber species included a remarkable amount of saturated (SFA) and monounsaturated fatty acids (MUFA) with a lower amount of polyunsaturated fatty acids (PUFA). Palmitic acid contributed the highest amount among all fatty acids. However, processing methodologies and environmental conditions might have influenced the overall fatty acid composition (Wen et al., 2010). Zhong et al. (2007) stated that other than the processing status, distribution of primary fatty acids in various tissues of *Cucumaria frondosa* was different. For instance, body wall tissues contained a low level of docosahexaenoic acid (DHA) compared to tissues of the whole body including the internal organs. However, compared to body walls, other organs such as the intestine and respiratory parts have a high amount of fatty acids, polysaccharides and glycosides (Bodbar et al., 2011). Meanwhile, lipids in sea cucumbers are rich in phospholipids, which account for more than 40% of total lipids (Lou, Wang, Liu and Xue, 2012). Therefore, a rich profile of numerous nutrients in sea cucumber may account for its use as a health-promoting food ingredient.

2.5 Industrial significance

In recent decades sea cucumber has gained much attention as a potential biomaterial with a wide range of applications in the food and medicinal sectors. This marine invertebrate also holds significant cultural importance primarily in China, Japan, Malaysia, Hong Kong, Singapore and Korea (Purcell, Williamson and Ngaluafe, 2018). Mainly, sea cucumber species are sold live, fresh, or frozen in these Asian seafood markets. However, it is popular as a dried product in the luxury seafood category (Xu et al., 2018). Besides being a delicacy due to its rich nutritional profile, sea cucumber is also playing a vital role

in industrial applications such as manufacturing of skincare creams, anti-wrinkle beauty products and numerous biomedical applications (Li et al., 2020). These various industrial applications have improved the aquaculture production of sea cucumbers compared to the wild catch. Therefore, cultured animals, such as *Holothuria scabra* (sandfish), have gained momentum in recent years and the industry is predicted to expand rapidly (Juinio-Meñez et al., 2017; Purcell et al., 2018). Figure 2.2 summarises the primary industrial applications of sea cucumbers.

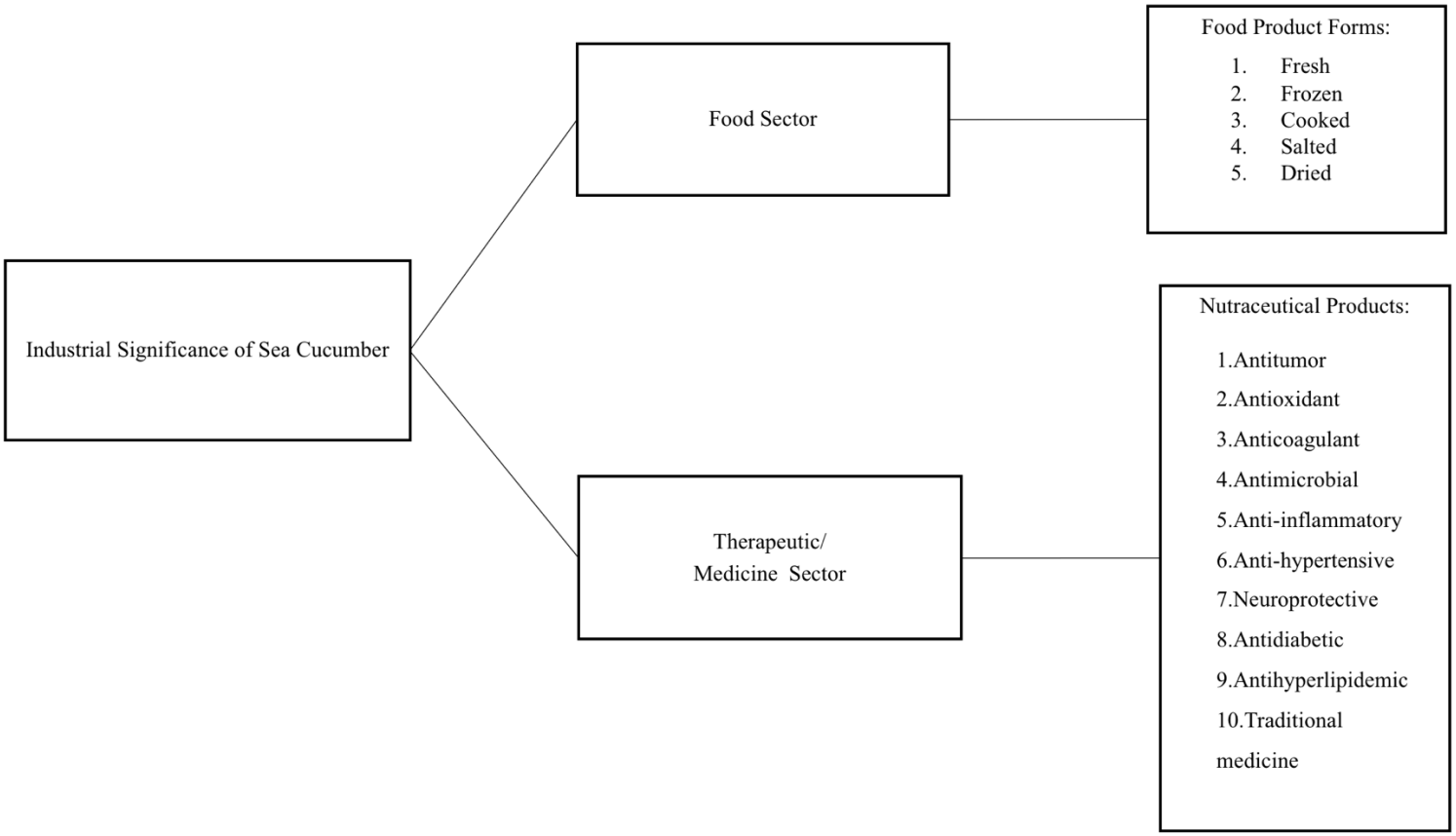


Figure 2.2 Primary industrial applications of sea cucumber

2.5.1 Food sector

Sea cucumber is a highly-prized delicacy, often served with sauced dishes as pieces or whole animal in most of the Asian cuisines (Song et al., 2020). The quality parameters, including size, color, shape and appearance of the products are vital factors for consumer perception of the food product. Sea cucumber is categorized according to species, abundance, appearance, odor, color, thickness of the body wall and primary market demand. For instance, red sea cucumber is more expensive compared to green or black as color variation affects the price and taste of the products (Oh, Ko, Lee, Heo and Jung, 2017). Hence, sea cucumbers are ranked as high, medium or low commercially viable seafood products (Zeng et al., 2018).

The major edible part of sea cucumber, the body wall, is sold directly as fresh products as well as processed, smoked and dried categories (Zhong et al., 2007). For instance, *Cucumaria frondosa* is used for producing a wide range of food products such as vacuum-packed or flash-frozen muscle bands and the remaining body wall is processed as boiled and dried product. These food products can be varied according to their regions. In Newfoundland (Eastern Canada), the same species of sea cucumber is used as cooked, salted and dried skin with the meat attached or separately processed muscles and skin in fresh and frozen forms (Toral – Granda, 2008). In most Asian countries, whole sea cucumber is processed as a high-value delicacy. However, chemical composition and nutritional value of sea cucumbers may vary due to different processing techniques (Zhong et al., 2007). Rehydration is required prior to consumption or cooking of most of the processed sea cucumber products (Moon, Kim, Chung, Pan and Yoon, 2014; Li et al., 2019). Li et al. (2019) demonstrated that commercial processing methods including vacuum

cooking and traditional processing (boiling and salting) could have a negative impact on the nutritional value as both methods significantly change the nutrient composition in the final ready to eat sea cucumber (*Apostichopus japonicas*) products.

Qi et al. (2017) stated that fresh sea cucumbers may be processed into instant and/or semi-dried products, which involves several cycles of heating and cooking to deactivate the autolyzing enzymes. Autolysis frequently occurs during transportation and processing, and as a post-harvesting processing step evisceration is conducted to prevent the quality deterioration caused by autolysis (Zhong et al., 2018). The processing discards of sea cucumber comprise of internal organs, including intestines, aquapharyngeal bulb, respiratory tract and gonads (Hossain et al., 2020). These by-products are usually employed as raw materials for fish meal or animal feed production, however, their potential use for higher value-added product development needs to be explored. By-products such as gonads are protein-rich resources with a content of 51.2% on a dry weight basis (Mamelona et al., 2010; Zhong et al., 2018).

The nutritional composition and molecular structures of bioactive compounds in sea cucumber processing discards and their properties are recently gaining attention as potential sources for novel products (Bordbar et al., 2011; Hamed, Özogul, Özogul and Regenstein, 2015). Hence, the recovery of sea cucumber processing discards may increase their potential for designing specific and personalized food products targeting special nutritional requirements. Besides, the myriad of beneficial dietary effects of sea cucumber products may provide a solution for addressing certain nutritional deficiencies (García et al., 2019).

2.5.2 Therapeutic and medicinal sector

In addition to their wide range of use as food ingredient, sea cucumbers are highly regarded as a valuable marine source with significant medicinal value (Zhong et al., 2007; Pangestuti and Arifin, 2018; Li et al.,2019). The importance of sea cucumber as a marine resource of pharmaceutical and possibly therapeutic ingredients has been extensively studied during the recent decades. In this connection, Bodbar et al. (2011) stated that the medicinal properties of sea cucumber functional components are associated with their bioactivities. A majority of the East Asian countries have long considered sea cucumbers as traditional medicine or folk remedy for the prevention and treatment of diseases such as asthma, hypertension, rheumatism, anemia and sinus cognition (Zhong et al., 2007). Moreover, sea cucumbers have been effective in nourishing the body, moistening dryness of intestines, treating stomach ulcers and specifically healing wounds in traditional medicine (Bodbar et al., 2011; Pangestuti and Arifin, 2018).

Presence of wide array of bioactive compounds such as triterpene glycosides, carotenoids, bioactive peptides, vitamins, minerals, enzymes, amyloses, fatty acids, cytotoxins, chondroitin sulfates, amino acids and phenols have led to the expansion of the applications of sea cucumber as a functional food ingredient (Silchenko et al., 2017; Xu et al., 2018). The curative power of this marine invertebrate is valued as a promising source that exhibits specific biological functions. Due to their diverse functionality, many studies have been conducted on exploiting the potentials of sea cucumbers as antioxidant (Zhong et al., 2007; Mamelona, Saint-Louis and Pelletier, 2010a; Zhou et al., 2016), anticancer (Li, Himaya and Kim 2013; Wijesinghe, Jeon, Ramasamy, Wahid and Vairappan, 2013;

Aminin et al., 2015), anticoagulant (Chen et al., 2012; Wu et al., 2012; Shi et al., 2016), anti-inflammation (Wang et al., 2016; Ding et al., 2018), antimicrobial (Mohammadizadeh et al., 2013; Mashjoor and Yousefzadi, 2017), antihypertensive (Pérez-Vega, Olivera-Castillo, Gómez-Ruiz, and Hernández-Ledesma, 2013; Ghanbari et al., 2015; Abedin et al., 2015), antidiabetic (Hu et al., 2014; El Barky, Hussein, Alm-Eldeen, Hafez and Mohamed, 2016; Wang, Wang, Huo and Li, 2016; Purwanto, Wiyasihati, Masyitha, Wigati and Irwadi, 2019; Gong et al., 2020), antihyperlipidemic (Shi et al., 2016) agents, among others. With the recent advancement of chemical analytical methods, sea cucumbers have been the subject of intense research in recent decades (Xu et al., 2018). To date, many research groups are still exploring the species-specific biological functions of sea cumbers. A list of biological functions and their bioactive compounds related to Atlantic sea cucumber (*Cucumaria frondosa*) are summarized in Table 2.1

Table 2.1 Bioactive properties and associated compounds derived from *Cucumaria frondosa*

Bioactive property	Bioactive compound	Reference
Antioxidant	Peptides, Phenolics, Organic extracts	Zhong et al. (2007); Mamelona et al. (2007); Yan et al. (2016)
Anti-inflammation	Fucosylated chondroitin sulfate	Liu et al. (2016)
Anti-diabetic	Fucosylated chondroitin sulfate, Phosphatidylcholine	Hu et al. (2014); Wang et al. (2016);
Anti-obesity	Phospholipid, Cerebrosides	Xu et al. (2015); Tian et al. (2016)
Antimicrobial	Glycosides, Fucoidan, Phenolic compounds, Peptides	Haug et al. (2002); Tripoteau et al. (2015)
Anti-cancer	Cerebrosides, Fucosylated chondroitin sulfate glycosides	Al Shemaili et al. (2014), Ustyuzhanina et al. (2017)
Anticoagulant	Fucosylated chondroitin sulfate, Fucoidan	Hu et al., (2014); Wang et al., (2016)

2.6 Protein isolates

Protein is an essential macronutrient that exerts numerous structural and functional properties in food systems. The isolation of protein-rich fractions from food matrix is a relatively recent approach used primarily for serving the techno-functional requirements of food systems (Alves and Tavares, 2019; Loveday, 2019). The empirical evidence from spontaneous coagulation of animal blood or rennet-induced milk proteins was believed to set the initial idea of recovering the protein-rich fractions from foods (Loveday, 2019). Protein isolates are the basic functional components of various high protein processed food products which can determine the textural and nutritional properties of foods and reported to be the most refined form of protein products (Foh, Wenshui, Amadou and Jiang, 2012; Panpipat and Chaijan, 2017).

Protein isolates are from different sources, including animal proteins (milk, muscle, egg, blood, insect and plant proteins (cereals, pseudocereals, seeds, legumes, tubers, oilseeds, algae) and fungal or microbial proteins (Loveday, 2019). According to their origin, the approach employed for the isolation of proteins may vary. The Osborne (1908) fractionation scheme for protein fractionation was based on plant proteins and the underlying conceptual approach was also the same for muscle/meat proteins (Strasburg et al., 2007). However, methods were tailored according to the final product. It is noteworthy that increasing interest in recent research on protein products has focused on developing animal-protein ingredients as supplement and substitutes for plant proteins (Alves and Tavares, 2019).

Muscle food proteins are categorized into three major groups as myofibrillar, stromal, and sarcoplasmic proteins (Matak Tahergorabi and Jaczynski., 2015). Myofibrillar proteins are the most significant components of muscle proteins and serve as the basic cellular unit of the muscle tissue (Matak et al., 2015). These myofibrillar proteins are major contributors to the functional properties of muscle food products. Zhang et al. (2016) investigated the microstructure of sea cucumber (*Stichopus japonicus*) and found that myofibrils present in body wall were more favourable for bound immobilized water and directly impact the water holding capacity of the muscle proteins. Compared to myofibrillar proteins, stromal proteins are predominantly composed of collagen and elastin and their contribution to the functional properties of food systems is less significant than myofibrillar proteins (Matak et al., 2015). However, significant number of studies have been conducted to determine the moisture absorption capacity of sea cucumber collagen. Zhu et al. (2012) reported that collagen from sea cucumber (*Stichopus japonicus*) has higher moisture absorption capacity compared to glycerol. These findings suggested that sea cucumber collagen has a potential to utilize in cosmetic industry.

The sarcoplasmic proteins contribute 25-30% to the total muscle proteins and it consist of myogen and enzymes. Both myogen and enzymes exhibit better water solubility compared to the other muscle proteins. However, their other functional properties such as gel-forming and water-holding abilities are much lower than myofibrillar and stromal proteins (Matak et al., 2015). The following scheme represents the classification of meat proteins after application of the Osborne fractionation principle (Loveday, 2019).

- Myofibrillar proteins are soluble at high salt concentration
- Sarcoplasmic proteins are soluble in water at low ionic strength

- Stromal proteins are insoluble in water or salt solution

2.6.1 Protein isolation methods

Among the various protein recovery methods, the pH-shift method dictated by protein solubility is regarded as an efficient recovery technique in protein research. Protein recovery from muscle proteins using pH-assisted process was initially proposed by Hultin and Kelleher (1999, 2000). The method of protein isoelectric behavior and subjecting the muscle source to pH changes was supported by the earlier discoveries of Meinke, Rahman and Mattil (1972).

According to the pH-shift method, protein from animal muscle tissues was isolated by shifting the pH from 3 to 11 and subsequently using isoelectric precipitation (ISP) as the final step of protein recovery (Abdollahi and Undeland, 2019). This method enables isolation of the protein with a high recovery yield with better functionality (Sahni, Sharma and Surasani, 2020). This pH-shift method is one of the efficient existing methods for preserving the functional properties of proteins with high nutritional profile from various sources (Panpipat and Chaijan, 2017). Notably, isoelectric point (pI) of fish muscle proteins were reported to have high recovery yields compared to other protein extraction methods such as surimi processing (Chen and Jaczynski, 2007). The pH shift method has been effectively applied for protein isolation from unconventional aquatic raw material including sea cucumber (*Stichopus japonicus*) gut materials (Du et al., 2019), different fish muscles (Davenport and Kristinsson, 2011; Foh et al., 2012; Shaviklo et al., 2016; Abdullahi, Rezaei, Jafarpour and Undeland, 2017), whole gutted fish (Marmon and Undeland 2010),

giant squid (*Dosidicus gigas*) (Zhang et al., 2017), blue mussels (Vareltzis and Undeland, 2012), whole Antarctic krill (*Euphausia superba*) (Chen, Tou and Jaczynski, 2009) and other aquatic processing by-products (Tahergorabi, Beamer, Matak and Jaczynski, 2012; Chomnawang and Yongsawatdigul, 2013; Panpipat and Chaijan, 2017; Surasani, Khatkar and Singh, 2017; Abdollahi and Undeland, 2019). Apart from the aquatic products, the isoelectric processing method has also been applied for recovering proteins from beef, and chicken processing by-products (DeWitt et al., 2002).

Initially, muscle proteins are solubilized at acid or alkaline pH. This takes advantage of the higher solubility of the protein in water, dilute salt as well as acidic and alkaline solutions (Du et al., 2020). Solubility plays a major role in separating muscle proteins from insoluble materials (Palafox et al., 2009). The solubility of proteins during the pH-shift process is dependent on the strong positive and negative charges of their side chains (Surasani, 2017). The acidic condition of the solution promotes the formation of hydronium ions (H_3O^+), and consequently, increased net positive surface charge of protein by accelerating protonation of negatively charged side chains (glutamyl or aspartyl residues). The addition of base (^-OH) to the solution facilitates the deprotonation of side chains, including lysyl, argininyl, cysteinyl, histidinyl, tyrosyl, tryptophanyl, or cysteinyl and improve the net negative surface charge on the protein (Figure 2.3) (Gehring, Gigliotti, Moritz, Tou and Jaczynski, 2011). Hence, the mechanism behind protein solubilization and precipitation depends on the charge distribution in protein molecules, the equilibrium of hydrophilic and hydrophobic interactions between protein and solvent molecules. For instance, myofibrillar proteins are extracted from the myofibrillar bundles and segments when the electrostatic interactions (protein-water) are more robust than hydrophobic

interactions (proteins-proteins) and solubilization of protein is favored (Gehring et al., 2011; Zayas, 2012; Surasani, 2017).

Solubilized proteins are selectively precipitated at their minimum solubility or the maximum precipitation level, which denotes the isoelectric point (Chen and Jaczynski, 2009). At their isoelectric point, proteins have a net zero electrostatic charge. Proteins precipitate at their isoelectric points, mainly due to the imbalance of hydrophobic and hydrophilic interactions between the molecules. When hydrophobic interactions between protein-protein are higher than their hydrophilic protein-water interactions, proteins start to aggregate (Matak et al., 2015).

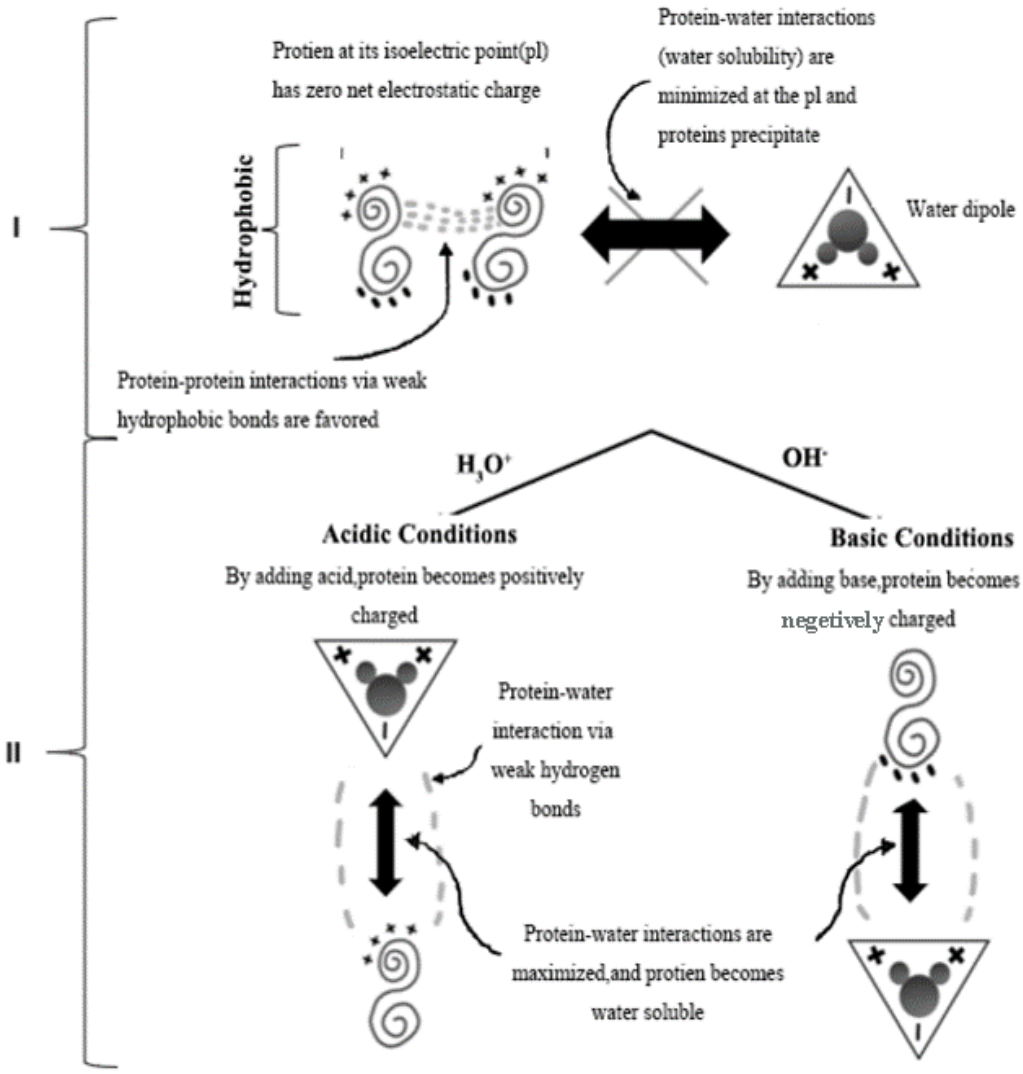


Figure 2.3 Biochemical changes associated with the pH-shifting protein recovery process I: Protein aggregation; II: Acid and alkali solubilization

[Source: adapted from Gehring et al., 2011; Matak et al., 2015]

The major advantage associated with ISP processing is promoting the selective, pH-induced water solubility of muscle proteins while removing other impurities, including bones, skin, scales, frames, connective tissues, and cellular membranes by centrifugation

(Tahergorabi et al., 2012; Du et al., 2020). The resultant protein isolate is highly functional and stable (Panpipat and Chaijan, 2017). The use of either an acidic solution or salt solution for protein solubilization was effectively applied in various studies. Previous studies such as protein extraction from sea cucumber (*Stichopus japonicus*) guts (Du et al., 2020), channel catfish (*Ictalurus punctatus*) muscles (Davenport and Kristinsson, 2011) and tilapia (*Oreochromis niloticus*) muscles (Foh et al., 2012) used acid solutions while salt solubilization method was employed to extract proteins from giant squid (*Dosidicus gigas*) (Zhang et al., 2017). Sodium hydroxide is generally used for protein solubilization at basic pH, and HCl is used for acid-base precipitation purposes (Tahergorabi et al., 2012).

However, simultaneous use of acid and salt treatment increases the recovery yield of proteins (Du et al, 2020). Most proteins are isolated from defatted raw materials (Timilsena, Adhikari, Barrow and Adhikari, 2016), and some studies use centrifugal separation of lipids from the aqueous solution (Du et al., 2020). Therefore, the removal of lipids or membrane phospholipids is considered as a vital step in the protein isolation process due to their susceptibility to oxidation (Kristinsson and Hultin, 2004).

Homogenization of the muscle followed by protein solubilization with high-speed centrifugation separates proteins from other constituents of the muscle and recovering proteins by isoelectric point is the major step included in the muscle protein isolation process (Kristinsson and Hultin, 2004; Chen and Jaczynski, 2007). Furthermore, quality of the raw material, processing parameters including process version of alkali or acid and solubilization/precipitation pH (Chomnawang and Yongsawatdigul, 2013; Abdollahi et al., 2016; Panpipat and Chaijan, 2017; Abdollahi and Undeland, 2019) and the amino acid composition may have a direct influence on the functional properties of protein isolates

(Foh et al., 2012). Table 2.2 summarises some of the most recent studies conducted based on the pH-shift methods for various aquatic sources.

Extreme pH level is known to retard microbial growth of the raw material (Tahergorabi et al., 2012). However, extreme pH conditions may negatively impact denaturation of muscle proteins, and as a consequence, functional properties might alter (Panpipat and Chaijan, 2017). In contrast, several studies have indicated that proteins having partially unfolded or folded structures possess better functional properties compared to the native protein due to flexibility of the molecule (Kristinsson and Hultin, 2004; Panpipat and Chaijan, 2017). Alkaline aided isolated protein isolates from sea cucumber (*Stichopus japonicus*) guts showed better functional properties related to solubility, gel-forming ability, emulsifying and foaming properties compared to sea cucumber gut powder (Du et al., 2019). In another study, the influence of exposing fish muscle protein isolates to different high and low pH treatments and the effect associated with protein solubility and molecular changes was examined (Kristinsson and Hultin, 2004). The findings indicated that the alkali and acid treatment greatly influences the solubility of muscle protein-hemoglobin due to the conformational changes in protein structure (Kristinsson and Hultin, 2004). A recent study evaluated the recovery of proteins from different fish species, including salmon (*Salmo salar*), cod (*Gadus morhua*) and herring (*Clupea harengus*) indicated a species-specific effect on the functional properties and yield of protein isolates (Abdollahi and Undeland, 2019).

Table 2.2 Protein extraction methods associated with the pH-shifting methods for various aquatic sources

Source	Protein extraction method	Tested properties of the isolated protein	References
Sea cucumber (*Stichopus japonicus*) guts	Alkali extraction and acid precipitation	Solubility Foaming capacity/ stability Oil/ water holding capacity Emulsifying capacity Thermal properties	Du et al. (2020)
Pangas (*Pangasius Pangasius*) processing waste	Alkali solubilization	Proximate composition Gel strength Texture profile analysis Color Moisture content Sensory analysis Emulsifying properties	Surasani et al. (2020)

		Water holding capacity Cooking yield Rheological measurements (visco-elastic behaviour)	
Large yellow croaker (*Pseudosciaena crocea*) roes.	Acid and alkali solubilization	Chemical composition Amino acid profile Solubility Molecular weight distribution Oil/ water holding capacity Emulsifying capacity Surface hydrophobicity Structural analysis	Du et al. (2020)
Salmon by-products	Alkaline solubilization	Water binding capacity Gel forming properties Color Molecular weight distribution	Abdollahi and Undeland (2019)

Cod by-products	Alkaline solubilization	Water binding capacity Gel forming properties Color Molecular weight distribution	Abdollahi and Undeland (2019)
Herring by-products	Alkaline solubilization	Water binding capacity Gel forming properties Color Molecular weight distribution	Abdollahi and Undeland (2019)
Bigeye snapper (*Priacanthus tayenus*) head by-product.	Acid and alkali solubilization	Solubility Surface hydrophobicity Gelation properties Texture analysis Color Molecular weight distribution Emulsifying capacity Foaming properties	Panpipat and Chaijan (2017)

Silver carp (*Hypophthalmichthys molitrix*)	Alkaline solubilization	Proximate composition Molecular weight distribution	Shi et al. (2017)
Antarctic Menhaden (*Brevoortia tyrannus*)	Alkaline solubilization	Proximate composition Molecular weight distribution	Shi et al. (2017)
Antarctic krill (*Euphausia superba*)	Alkaline solubilization	Proximate composition Molecular weight distribution	Shi et al. (2017)
Giant squid (*Dosidicus gigas*)	Alkali solubilization	Structural analysis Molecular weight distribution Solubility	Zhang et al. (2017)
Tilapia frame by-products	Alkali solubilization	Solubility Gel forming ability	Chomnawang and Yongsawatdigul, (2013)
Blue mussel (*Mytilus edulis*) meat and whole mussel	Acid and alkali solubilization	Solubility Nutritional composition	Vareltzis and Undeland (2012)

		Stability over lipid oxidation and proteolysis	
Tilapia (*Oreochromis niloticus*) muscles	Acid and alkali solubilization	Molecular weight distribution Thermal properties Solubility Foaming capacity/ stability Oil/ water holding capacity Emulsifying capacity Bulk density Viscosity	Foh et al. (2012)
Striped bass (*Morone saxatilis*)	Alkali solubilization	Gelation properties Color Texture analysis Mineral content Oxidative rancidity	Tahergorabi et al. (2012)

Saithe (*Pollachius virens*) muscles	Alkaline solubilization	Water binding capacity Gel forming properties Emulsification Foaming	Shaviklo et al. (2012)
Blue mussel (*Mytilus edulis*) meat and whole mussel	Acid and alkali solubilization	Solubility Nutritional composition Stability over lipid oxidation and proteolysis	Vareltzis and Undeland (2012)
Channel catfish (*Ictalurus punctatus*) muscles	Acid and alkali solubilization	Gel formation properties Water-holding capacity Cooking loss	Davenport and Kristinsson (2011)
Whole gutted herring	Acid and alkali solubilization method	Color Nutritional composition Gel strength	Marmon and Undeland (2010)
Antarctic krill (*Euphausia superba*)	Acid and alkali solubilization	Amino acid profile Mineral content	Chen, Tou and Jaczynski (2009)

		Recovery yield	
Jumbo squid (*Dosidicus gigas*)	Acid and alkali solubilization	Solubility Molecular weight distribution	Palafox et al. (2009)
Cape hake (*Merluccius capensis*) by-products (sawdust)	Alkali solubilization	Proximate analysis Gel forming ability	Pires, Batista, Fradinho and Costa (2009)
Rainbow trout (*Oncorhynchus mykiss)* processing by-products	Acid and alkali solubilization	Amino acid profile Proximate analysis	Chen, Tou and Jaczynski (2007)

Shi et al. (2017) analyzed the pI processing of carp, chicken, menhaden, and krill based on their mass balance of recovery fractions and revealed that despite the species, pH assisted protein isolation process can be applied to recover nutrients from underutilized materials. Hence, the pH-shifting method could be an effective approach for upgrading the low-value processing discards.

In general pH-shift method has several advantages including high recovery rate of protein, effective on underutilized/waste sources, improve the functional properties of food products, increase the stability of the protein by removing most of the membrane lipids, and enhance the nutritional value of the food products (Shaviklo and Etemadian, 2019).

2.6.1.1 Limitations

One of the major limitations associated with the pH-shift method in muscle protein isolation is the lack of removal of heme-pigments, including hemoglobin and myoglobin (Abdollahi, Marmon, Chaijan and Undeland, 2016). It can negatively affect the stability and sensory qualities of the final product. Therefore, the development of novel products using muscle protein isolates or the addition of the isolates to the food products should proceed after conducting a series of physicochemical analyses (Shaviklo, Arason, Thorkelsson, Sveinsdottir and Martinsdottir, 2010).

2.6.2 Applications of protein isolates

Physicochemical properties of protein isolates directly determine their potential applications in food systems. Recent studies have indicated that protein isolates have the potential to be used as an ingredient for manufacturing different types of food product (Foh et al., 2012). Numerous desirable functional properties of protein isolates compared to the

native proteins have expanded their applications over their native counterparts (Pires et al., 2009). Furthermore, pI recovered proteins can be used as additives as well as major ingredients in food formulations (Shaviklo and Etemadian, 2019). Most of the pI recovered proteins are known as intermediate raw materials in food formulations and serve as carriers for high nutritious dietary compounds. They could also serve as emulsifiers and nutritive binders (Shaviklo and Etemadian, 2019).

The protein isolates could be used as a base or bulk ingredient to produce functional food products that enable the maximum utilization of underutilized sources, including seafood and other muscle food processing by-products (Matak et al., 2015). The use of pI recovers protein to develop food products will diversify the utilization of muscle foods and meat processing by-products while enhancing protein availability for direct consumption (Palafox et al., 2009; Matak et al., 2015). Surasani et al. (2020) studied the application of protein isolates from by-products of Indian major carps and Pangas in the food systems to investigate their functionality along with quality characteristics and acceptability of the final product (fish sausages). They found that the addition of Pangas protein isolates into fish sausages significantly enhances the quality attributes as well as their nutritional value without affecting their sensory properties (Surasani et al., 2020). A previous study in developing a frankfurter type fish sausage with alkali isolated proteins from Cape hake by-products was shown to have better sensory and textural properties compared to commercially available sausages (Pires et al., 2009).

Ibrahim (2015) incorporated fish protein isolates recovered from underutilized Nile Bolti fish into beef and fish balls to examine their nutritional value and functional properties. The findings of this study indicated that fish protein isolates improved the

essential amino acid index and functional properties of formulated beef and fish balls compared to the controls without fish protein isolates. In another study, tuna protein isolates were incorporated into the formulation of silver carp burger to determine their influence on storage on the sensory quality of the product (Shaviklo et al., 2016). The results indicated that inclusion of 20% of tuna protein isolate rendered better sensory quality and acceptability to burgers compared to the commercially formulated burgers for six months during frozen storage.

However, the use of muscle protein isolates or their addition to the food products may be detrimental to sensory properties of the final product as well as consumer acceptability. Therefore, the amount of protein isolates incorporated into food systems should be assessed frequently in product development studies from the designing stage related to protein isolates. Moosavi-Nasab, Mohammadi and Oliyaei, (2018) evaluated the physicochemical and sensory properties of sausages developed by incorporating various concentrations of protein isolates from lantern fish (*Benthosema pterotum*) and reported the highest overall acceptability of samples containing 2% protein isolates. Foh et al. (2012) and Kristinsson and Liang (2006) reported similar results stating that 2% protein isolates were preferred over higher concentrations of protein isolates in terms of sensory attributes. Furthermore, the sensory properties of meat protein isolate incorporated food products correlated with the amount of heme protein and lipid contents (Moosavi-Nasab et al., 2018). The presence of pigments is a critical factor for quality attributes and consumer perception. Kristinsson et al. (2005) reported that the use of basic pH ensures the reduction of heme proteins as well as preserving the essential amino acids in adequate quantities in the resultant product. Therefore, subsequent tailoring of processing parameters to yield a

better-quality product is necessary. This requires a better understanding of the technological applications of protein isolates and assessment of their potential application for commercial scale production of novel and value-added food products.

2.6.3 Identification and Quantification of Isolated Proteins

Determination of protein content is often expressed with the amount of nitrogen present in a particular sample. The Kjeldahl method is frequently used in this regard. However, it is a tedious and time-consuming method for routine analysis of proteins (Zheng, Wu, He, Yang and Yang, 2017). Due to the complicated nature of the proteins, more advanced robust methods are introduced to identify and quantify the proteins beyond the elementary protein concentration determination methods (Sapan et al., 1999). Accuracy and sensitivity of the new techniques are comparatively higher than primary methods as they are capable of providing detailed information about distinctive properties of different proteins (Kambhampati, Li, Evans and Allen, 2019).

Furthermore, identification and quantification of proteins plays an important in proteomics. According to Gevaert and Vandekerckhove (2000), proteome analysis can be categorized into four steps. These include: (1) purification of proteins from complex mixtures using a fast and simple method, (2) identification of structural formation of proteins of interest using a rapid and sensitive method, (3) evaluation of extending protein or DNA sequence using databases and (4) use of computer algorithms to link the DNA sequence with the structural information of the interested protein. Based on these four, the first step is to use a simple and fast technique for purification or isolation of a protein of interest from complex mixtures (Gevaert and Vandekerckhove, 2000). The basic analytical

procedures to locate the desired protein in a complex mixture might include spectrophotometric or chromatographic methods with gel electrophoresis and other relevant techniques (Simonian and Smith, 2006). Therefore, establishing methods to improve the accuracy of protein quantification at each step of the protein isolation process is mandatory (Simonian and Smith, 2006).

2.6.3.1 Colorimetric methods

Colorimetric determination of protein concentration is commonly used in research laboratories and considered as a non-destructive and rapid methods for protein detection (Simonian and Smith, 2006). Most of the colorimetric methods are based on spectrophotometric analysis followed by a chromophore addition (Kambhampati et al., 2019). The well-known colorimetric methods for protein quantification are (a) Bradford assay, (b) biuret method, (3) Lowry method and (d) bicinchoninic acid (BCA) assay (Sapan et al., 1999).

Bradford assay or Coomassie Blue G-250 dye-binding assay is a rapid and sensitive assay that is widely used to determine the protein concentration in a wide variety of samples (Sapan et al., 1999). The assay quantifies the binding of the dye to unknown proteins and comparing it with the binding to a known standard protein sample followed by reading the absorbance of the dye-protein complex at 595 nm (Simonian and Smith, 2006). However, there are few limitations associated with the Bradford assay including irreversible denaturation of used proteins (Sapan et al., 1999).

The Biuret method is empirical to a reaction that produces a purplish-violet coloration, with a maximum absorbance at 545 nm, when peptide bonds react with cupric

ions at alkaline pH. Cupric ions form a coordination complex with the NH groups of the peptide bonds of the protein is responsible for this coloration (Sapan et al., 1999; Zheng et al., 2017). Compared to other colorimetric methods, the biuret method has better precision and less interference with other non-protein substances (Arora et al., 2018). The intensity of the color is proportional to the protein content in the test sample (Zheng et al., 2017). This method is simple and reliable in which all proteins react similarly. Hence, the purity of the protein of interest is important for obtaining accurate and reproducible results (Sapan et al., 1999).

The Lowry method is reported to be more sensitive than the Biuret method and uses mainly Folin-Ciocalteu reagent (Sapan et al., 1999). This method is based on the peptides bonds of proteins that react with the Folin reagent under alkaline conditions in the presence of cupric ions. Thus, Lowry's reaction is amplifying the biuret reaction by subsequently reacting with the Folin- Ciocalteu reagent (Folin and Ciocalteu, 1927). The reaction produces an intense blue colored complex that depends on the content of aromatic amino acids, mainly tyrosine and tryptophan (Waterborg, 2009). The absorbance of the color complex so formed is read at 750 nm in order to determine the protein content of the sample (Simonian and Smith, 2006). The Lowry method is sensitive to the amino acid composition of the sample and sensitive enough to evaluate protein concentrations in the range of 0.01-1.0 mg/mL (Waterborg, 2009). However, it is difficult to obtain absolute protein concentration using only the Lowry method.

Similar to the Lowry method, the fundamental concept of BCA assay is the conversion of cupric ions to cuprous ions under alkaline conditions. The reaction between cuprous ions and BCA produces an intense purple color complex that can be detected at

562 nm (Walker, 2009). BCA assay is relatively easy to perform and no effect from the detergents and other denaturing agents that can interfere with the Lowry method could be noted (Simonian and Smith, 2006; Walker, 2009). Nevertheless, the main drawbacks of the BCA assay include the influence of protein-to-protein variation and sensitivity to the presence of reducing sugars such as fructose and lactose to the BCA reaction (Sapan et al., 1999; Walker, 2009).

However, most of the colorimetric assay results rely on the comparison with known protein standards and depend on the quality of the sample (Daniel and Edelstein, 1991; Sapan et al., 1999; Simonian and Smith, 2006; Walker, 2009). Each assay has both advantages and limitations according to the respective reagents and their chemical reaction with other substances. Most of the spectrophotometric methods are limited to detect only soluble proteins and their accuracy depends on the purity of the sample (Kambhampati et al., 2019). Sapan et al. (1999) stated that biuret, Lowry, and BCA methods overestimate the protein concentration. Thus, selecting the appropriate standards is crucial to ensure the precision of protein concentration of the desired sample. Furthermore, using validated procedures for protein determination may provide more accurate final results.

2.6.3.2 SDS-PAGE

Electrophoresis is a classic platform in proteomics which separates the analytes based on their charge-to size ratio (Jorrin-Novo, Komatsu, Sanchez-Lucas and Rodríguez de Francisco, 2019). Different types of electrophoresis methods are available for protein analysis. Most of these methods, including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) are based on the ability of proteins to migrate through the

influence of an electric field. Furthermore, SDS-PAGE is considered as a primary analytical technique that can resolve the individual components of a complex protein mixture (Hong, Fan, Chalamaiah and Wu, 2019). SDS-PAGE is employed in a myriad of fields, including environmental, biomedical, forensic, and clinical areas for analyzing the size, amount, purity, and the isoelectric point of proteins and peptides (Dawod, Arvin and Kennedy, 2017). Furthermore, this technique is often used for fractionation and quantification of proteins, possibly in association with either mass spectrometric identifications or immunological tests (Magdeldin et al., 2014). SDS is a detergent with a negative charge and has the capability of binding to the soluble protein molecules in aqueous solutions over a wide pH range (Goetz et al., 2004).

SDS binds along the polypeptide chains, and the amount of bound SDS is proportional to the size of the molecule. However, one drawback of the method is that SDS selectively binds to the protein molecules with primary structures. Thus, complex proteins (secondary, tertiary or quaternary structures) should be denatured prior to their analysis by using reducing agents such as dithiothreitol or 2-mercaptoethanol (Goetz et al., 2004; Magdeldin et al., 2014). The length of the reduced SDS-protein complex is proportional to the molecular weight of the tested sample. The use of known molecular markers facilitates estimation of the molecular weight of polypeptides present in the protein.

SDS-PAGE method is a low-cost and reproducible tool to characterize, compare, and quantify proteins. However, with the newly emerging techniques such as protein sequencing, amino acid compositional analysis, peptide profiling may make the SDS-PAGE method less attractive (Bhagwat and Dandge, 2016). Therefore, SDS-PAGE is often

confined to preparative purification procedures of protein for other subsequent microchemical analyses (Doonan, 1996).

2.6.3.3 Chromatographic methods

It is difficult to avoid loss of material during protein preparation and purification for protein quantification. The interference of other non-protein materials may influence the accuracy of the measurements (Kambhampati et al., 2019). The use of chromatographic techniques facilitates evaluation of protein levels based on amino acid composition analysis as they provide a more accurate outcome compared to other quantification methods by mitigating most of the limitations associated with spectrophotometric and colorimetric methods.

Chromatography is a proven technique for separating and analyzing the components of a complex mixture and is most effective when evaluating biological extracts (Miller, 2005). Chromatographic separation is often linked with the migration of the components through a column (Engelhardt, 2012). These techniques are based on principles such as adsorption, partition, ion-exchange or molecular exclusion for separation purposes (Table 2.3).

Table 2.3 Properties of protein used in different chromatographic methods

Type of Chromatography	Property of Protein
Size exclusion chromatography	Size and shape
Ion exchange chromatography	Net charge and distribution of charged groups
Reversed phase chromatography	Hydrophobicity
Hydrophobic interaction chromatography	Hydrophobicity
Immobilized metal ion affinity chromatography	Metal binding ability
Covalent chromatography	Content of exposed thiol groups
Affinity chromatography	Biospecific affinities for ligands
Immunoadsorption	Antigenicity
Chromatofocusing	Isoelectric point

Adapted from: Janson, (2012)

The chromatographic methods are categorized into different types, including paper chromatography, thin-layer chromatography, gas chromatography, liquid chromatography, high-performance liquid chromatography, ion-exchange chromatography and gel filtration chromatography. In general, chromatographic methods consist of two phases known as the stationary and mobile phase. The stationary phase is referred to as the static portion, while the mobile phase is the moving portion (Gooding and Regnier, 2002). Separation efficiency depends on protein distribution between the stationary and mobile phase (Engelhardt, 2012). The typical nature of the mobile phase is generally a liquid or gas. Vertical columns

made of glass, plastic or stainless steel are used in general to pack the stationary phase (Janson, 2012). As an effective method for protein isolation, chromatographic analysis is frequently conducted, followed by centrifugation or filtration steps. For the preparation of columns, a wide range of chromatographic packing materials are used. In commercial level applications, gel filtration media, ion exchangers, reversed-phase packing, hydrophobic interaction adsorbents, affinity chromatography adsorbents are widely used as packing material (Sun, Chance, Graessley and Lohse, 2004; Jason, 2012).

2.6.3.3.1 High performance liquid chromatography (HPLC)

HPLC is an efficient analytical method in which the stationary phase is fabricated by the microparticulate column packing material and separation efficiency increases according to the size of the particles in the stationary phase (Barnes, 1992). Narrow columns (diameter of 2-8 mm) should be packed uniformly with particles of less than 50 µm. Commonly, spherical or irregular shaped porous silica particles are used to acquire the uniformity (Barnes, 1992; Engelhardt, 2012). The high pressure (10-400 atm) with a flow rate of 0.1 and 5 cm/ sec or more is essential for maintaining the velocity of the mobile phase (Engelhardt, 2012). A constant flow rate is a mandatory requirement to maintain the reproducibility of HPLC results. Two types of elution are associated with HPLC, namely isocratic elution and gradient elution. The nature and composition of the mobile phase determines the type of elution. Isocratic elution has a constant mobile phase and if the composition is altered during the separation, it is referred to as gradient elution (Barnes, 1992).

Reversed-phase-high performance liquid chromatography (RP-HPLC) is the classical method in the characterization and purification of peptides, including their extraction from marine sources (Pal and Suresh, 2016). Numerous studies extensively use the RP-HPLC method for separation of low molecular weight peptides and profiling of amino acids.

In addition to HPLC, most common compositional analysis of amino acid includes (a) ultra-high performance liquid chromatography (UPLC/UHPLC), (b) gas chromatography (GC) or capillary electrophoresis (CE) coupled to detection by absorbance, and (c) fluorescence or mass spectrometry (MS) (Kambhampati et al., 2019). Notably, mass spectroscopy (MS) analysis is manifested as a high-throughput identification method in proteomics (Gevaert and Vandekerckhove, 2000). MS technology measures the mass-to-charge ratio of the molecules and performs two main functions, namely ionization and mass analysis. Ionization techniques can be divided into two categories as electrospray ionization and matrix-assisted laser desorption/ ionisation (MALDI) coupled with mass analyzers and detectors (Wilkins et al., 2006). In light of sophisticated technologies, novel MS approaches for protein and amino acid analysis are moving towards using of biomarkers to detect and quantify the specific proteins and their amino acid compositions (Wilkins et al., 2006). The comprehensive protein analysis using chromatographic methods depends on the proper separation of intact amino acids and their resolution (Melfi Nardiello, Natale, Quinto and Centonze,, 2019). Consequently, it determines the accuracy of the final measurements.

More than one separation techniques are often combined to minimize the sample complexity before analysis (Wilkins et al., 2006). Hydrolysis or digestion step of the

samples generally performs before the separation procedure. It is crucial to break the high molecular mass proteins to small fragments in order to facilitate their identification. This fragmentation process describes the "shotgun" proteomics approach where a complex mixture of proteins is finally broken down into small peptides followed by analysis of single-stage MS or MS/MS (Wilkins et al., 2006). The basic types of mass analyzers used in protein identification include quadrupole, ion-trap, time-of-flight (TOF) and FT ion cyclotron resonance (ICR) (Wilkins et al., 2006).

2.6.4 Physicochemical and functional properties of food proteins

Characteristics of proteins that determine their utilization as ingredients in food are known as functional properties (Kristo and Corredig, 2015). Besides nutritional relevance, the physicochemical properties of proteins have a direct influence on the functionalities of the food systems from processing to consumption (Kinsella, 1982). The functional role of proteins in food is mainly dependent on their concentration, processing history and environmental conditions. The structural versatility of protein determines the task performed by them in a specific food application (Foegeding, 2015; Alves and Tavares, 2019). Thus, the type of protein is selected depending on the type of functionality required for the food system.

Proteins are one of the structural building blocks of the food matrix that can influence the bioavailability of nutrients and other bioactives (Foegeding, 2015; Kristo and Corredig, 2015). The structure-function relationship of proteins has a profound effect on the different aspects of protein functionality. The most common functions of the food proteins include solubility, emulsification, gelation, foaming, water binding, and heat

stability that play a significant role in determining desirable textural characteristics of the food (Foegeding and Davis, 2011). Besides texture, other hedonic factors such as color, flavor and organoleptic attributes are also reflected in the functional properties of food proteins (Kinsella, 1982). Thus, functional properties of food protein make a remarkable contribution to the quality attributes of food systems. Furthermore, fundamental factors that determine the functional properties of food proteins include structural and conformational characteristics as well as physical and chemical parameters. These include amino acid composition and sequence, hydrophilicity / hydrophobicity ratio, particular distribution of charges, size, shape, flexibility/rigidity, inter- and intramolecular subunit cross-links, secondary, tertiary and quaternary arrangements of polypeptides or three-dimensional structure of the food proteins (Wanasundara and Shahidi, 1994). From a molecular consideration, functional properties of proteins are manifested by two phenomena, namely protein surface-related and hydrodynamic properties (Hettiarachchy and Ziegler, 1994).

Determining functional properties of proteins requires structural analysis, molecular properties, interfacial properties, and simple quantitative measurements including numerical ratings to determine the specific function upon formulation of food products (Foegeding and Davis, 2011). However, Harper, Hewitt and Huffman (2020) highlighted the importance of studying pure proteins in simple systems and investigating the commercial proteins in food systems related to their functionality. Furthermore, the functional behavior of food proteins and their fundamental relationship between conformational properties need to be fully understood to maximize their utilization in the food industry (Kristo and Corredig, 2015). Table 2.4 presents some of the typical functional properties that are conferred to foods by proteins. Besides developing standardized testing

methods for measuring functional properties, it is necessary to focus on strategies to enhance protein functionality, including novel processing techniques and exploring underutilized food proteins.

Table 2.4 Functional attributes of proteins important in food applications[1]

Functional attribute	General property	Mode of action	Food system
Solubility	Hydration	Protein solvation	Beverages
Emulsification	Surface related	Formation and stabilization of fat emulsions	Cakes, Sausages, Soup, bologna
Foaming ability	Surface related	Entrap gas by formation of stable films	Whipped toppings, ice creams, icing, desserts, bakery products
Water holding capacity	Hydration and binding ability	Entrap of water without dripping and hydrogen bonding of water	Meat products, bakery products
Fat absorption	Binding ability	Binding with lipid molecules	Meat products, bakery products
Gelation	Rheological property	Protein matrix formation and setting	Meat products, dairy products (cheese, curd)
Viscosity	Rheological property	Thickening and water binding	Gravies, soups

Cohesion-adhesion	Structural properties	Act as adhesive material	Meat products, bakery products
Flavour-binding	Surface related	Adsorption, entrapment, release	Meat products, breaker products

[1]Adapted from Kinsella (1982); Kristo and Corredig (2015)

2.6.4.1 Color

The first quality attribute of food that attracts the attention of consumers is color, as the appearance of the food product plays a vital role in its acceptability (Wu and Sun, 2013). Color is the primary visual parameter that depends on both physical and psychological factors related to the food product that can impacts human perceptions (Hutchings, 2011). The appearance of food is the major immediate criterion in making purchasing decisions which is evaluated by their size, shape, form and color (Kays, 1999). However, color is the most important attribute that has a direct association with evaluating food quality parameters such as freshness, desirability, maturity and safety (Wu and Sun, 2013). Thus, color is referred to as an indicator for determining the product quality, and color evaluation assessments are based on objective measures (Pallottino et al., 2010; Wu and Sun, 2013). Quantitative measurements of the color include: (a) hue or spectral color which is an attribute of a visual sensation of an area that appears to be red, green, blue or intermediate color between these (b) saturation which is the colorfulness of the objective related to the strength or intensity (3) brightness or illuminance that appears to be white or highly transmitted from the object (Fairchild, 2013). The color measurements can be determined either by visual (human inspection) or using traditional instruments such as colorimeter and spectrophotometer (Wu and Sun, 2013).

2.6.4.2 Solubility

Solubility is a vital index in functional properties that determines the use of proteins in various food products. Solubility predominantly affects the emulsifying capacity, foaming and gel-forming ability of food proteins (Sikorski, 2001). The solubility behavior

of proteins under various conditions to perform these functions is required to evaluate the potential applications of proteins in food products. As one of the most crucial attributes, soluble proteins provide a homogeneous distribution of molecules in colloidal systems and improve the interfacial properties (Thiansilakul, Benjakul and Shahidi, 2007). The decrease in solubility is correlated with protein denaturation and aggregation (Thiansilakul et al., 2007). It may result in an unattractive appearance as well as an undesirable mouthfeel (Kristinsson and Rasco, 2000). Hence, the degree of protein insolubility is an indicator of protein denaturation or regarded as the measurement of loss of solubility. Denaturation of proteins may cause several changes in functionalities, including foaming, emulsification and gelation (Kinsella, 1982).

Equilibrium between repulsive electrostatic and attractive hydrophobic forces determine the solubility of proteins (Kristinsson and Hultin, 2004). Hydrophobic interactions include protein-protein interactions that decrease the solubility of the protein and promote the aggregation of proteins. Electronic repulsions are mainly due to the ionic residues on the surface of the protein molecules that contribute to improving the solubility (Kristinsson and Rasco, 2000). Hence, higher ionic interactions and lower hydrophobicity of the protein molecules would result in higher solubility of proteins (Nakai, 1983). Furthermore, molecular characteristics such as the content of hydrophobic and hydrophilic amino acids and their distribution in the primary structure also influence the solubility of protein (Kinsella, 1982).

Protein solubility is strongly related to the ISP and the change of the pH (as discussed in section 2.2.1). The loss of net protein charge at ISP is the predominant factor that is considered at the crucial stage of preparation and processing of protein ingredients

(Kristinsson and Rasco, 2000; Sikorski, 2001). At neutral or isoelectric pH, protein-protein electrostatic repulsion interactions promote the conditions for aggregation and precipitation by reducing the repulsive electrostatic forces of the protein. This condition ultimately decreases the solubility of protein (Du et al., 2020). Proteins at pH values higher than their pI carry a net negative charge while proteins with lower pH values compared to the isoelectric pH have a net positive charge. These charges of proteins facilitate their interactions with water molecules and enhance solubilization of proteins. Abdollahi and Undeland (2019) stated that increasing electrostatic charges by changing the pH of the solution subsequently improves water-protein interactions and increases the solubility of the protein of interest. The solubility of a protein as a function of pH is often a V- or U-shape curve indicating minimum solubility in the vicinity of the isoelectric pH (Chen and Jaczynski, 2007).

Dissociation of aggregates due to the changes in charge distribution and unfolding of the native structure of proteins may accelerate the solubilization process (Nakai, 1983). Physicochemical nature of proteins can directly determine the interaction of proteins with the surrounding solvent molecules. Physicochemical nature of the protein may be influenced by the folding pattern of the polypeptide chain as the extent of exposure of hydrophobic and hydrophilic residues on the surface of the protein depends on their degree of unfolding (Hettiarachchy and Ziegler, 1994). The relationship between protein solubility and charge distribution of the protein molecule has been extensively studied (Nakai, 1983; Matak et al., 2015; Panpipat and Chaijan, 2017; Du et al., 2020). According to Nakai (1983), the degree of molecular unfolding is crucial for protein solubility has a direct impact on the availability of hydrophobic sites. Thus, hydrophobic parameters including surface

hydrophobicity, aromatic amino acid contents were crucial for determining the correlation of physicochemical properties with the protein solubility (Nakai, 1983).

In their native state, proteins exhibit weak solubilization characteristics. For instance, intact fish myofibrillar protein shows weak solubility in water, and enzymatic modifications can enhance its solubility over a wide range of pH (Kristinsson and Rasco, 2000). The enzymatic hydrolysis results in the enhancement of solubility by digesting the intact proteins into low molecular weight peptides, thus improving hydrophilicity by exposing ionizable groups (amino and carboxyl) in amino acids (Shahidi, Han and Synowiecki, 1995; Kristinsson and Rasco, 2000). Hydrolyzed products of proteins impart numerous desirable functional properties, including solubility, as well as enriching the nutritional value (Panyam and Kilara, 1996). These beneficial characteristics widen the application of proteins in different food systems. In addition, various treatments, including heat, alkali, and acidic modifications, markedly affect the solubilization of proteins, mainly altering the configuration of proteins (Kristinsson and Rasco, 2000). The type of modification and conditions for processing protein is predominantly decided by the requirement of the food system and commercial interest (Panyam and Kilara, 1996).

The ionic strength is also a determinant factor in the solubilization of protein. In particular, muscle proteins, including myofibrillar proteins, are water-insoluble at their physiological ionic strength and soluble at low ionic strength (Chen and Jaczynski, 2007). However, the solubility of protein decreases with increasing ionic strength. The use of a neutral salt solution of relatively high ionic strength (0.5-1 M) may increase the solubility of structural proteins (Kristinsson and Rasco, 2000). The phenomenon behind the solubilization of proteins in neutral salts is the salting-in effect. The ions of the salt solution

interact with the charges of proteins and promote protein solubility (Chen and Jaczynski, 2007). In contrast, at high salt concentrations (higher than 1M), water molecules are not available for protein solvation as ionic strength increases interaction of water molecules with the solvating ions. This process is known as salting-out and the resultant protein gets aggregated and precipitates due to stronger protein-protein interactions compared to protein-water interactions (Duong-Ly and Gabelli, 2014).

2.6.4.3 Water holding capacity

The ability of the proteinaceous food matrix to imbibe and retain endogenous or added water against gravitational force is referred to as water-binding or water-holding ability (Shahidi 1995; Kristinsson and Rasco, 2000). Water holding capacity is inversely related to the solubility of the protein. However, the mechanism of water holding in the food matrix is only partially understood (Ertbjerg and Puolanne, 2017).

Water holding capacity is an essential quality attribute and essential for other sensory parameters of the final product (Shahidi 1995; Bao and Ertbjerg, 2019). This quality attribute may greatly influence food texture, including juiciness, tenderness related to palatability, as well as flavor, color, as well as cooking and drip losses (Ertbjerg and Puolanne, 2017). For instance, water absorption is a crucial factor in processing muscle foods and baked doughs as it has a direct impact on developing the desirable food texture (Haque, Timilsena and Adhikari, 2016). Preventing water being expelled from the proteinaceous food matrix primarily depends on the water-protein interactions (hydrophilic interactions), the configuration characteristics of proteins present and other extrinsic factors, including pH and temperature. For instance, factors affecting the water-holding

ability of muscle food products include various aspects such as the electrostatic repulsion and the surface area of the proteins interacting with sarcoplasm (Ertbjerg and Puolanne, 2017).

The hydrophilicity of the protein is determined by the interactions between polar amino groups (amino, carbonyl, hydroxyl and sulfhydryl groups) and water molecules (Chen and Jaczynski, 2007). Conformational changes of proteins due to extrinsic environmental factors may influence the nature and the availability of the hydration sites of the protein and charge distribution (Pires and Batista, 2013). Alterations of the pH of the protein solution may lead to configuration changes by exposing or concealing water binding sites due to their effect on ionizable groups present in proteins (Haque et al., 2016). Thomsen and Zeuthen (1988) reported that water holding capacity improves with increasing pH of the meat. The authors concluded that an increase in meat pH could be responsible for the decrease in cooking loss.

Furthermore, heat treatments of protein solutions also affect the water retention ability of the protein. As a consequence of heating, the globular conformation of protein may transform to the random coil state and unfolded protein binds more water molecules than when in its globular form (Chen and Jaczynski, 2007). Besides, some researchers investigated the effect of oxidation of meat proteins on water holding capacity (Ertbjerg and Puolanne, 2017; Bao and Ertbjerg, 2019; Zhang et al., 2020). The oxidation damage of muscle proteins resulted in alteration of proteins structure, changes in the net charge of proteins due to losing or gaining of protons of amino acids during oxidation (Zhang et al., 2020). However, the effect of oxidation on water holding capacity of protein is a complex and underexplored subject area (Ertbjerg and Puolanne, 2017; Bao and Ertbjerg, 2019).

Utrera and Estevez (2012) stated that an increase in net charge in muscle proteins causes the expansion of myofibrils and improves their water holding capacity. In contrast, Traore et al., (2012) and Chen, Zhou and Zhang (2015) showed that high drip loss in muscle protein (pork) could be related to the oxidation that decreases the water holding capacity of pork.

The incorporation of hydrolyzed proteins into muscle foods resulted in excellent water holding capacity as well as retarding the oxidation damage (Ambigaipalan and Shahidi, 2015). As the hydrolysis process advances, the polar groups in amino acid side chains are exposed and able to interact with water in the medium (Kristinsson and Rasco, 2000). Extensive research has been conducted to evaluate the ability of protein hydrolysates that enhance the water-holding ability of muscle foods. Cumby, Zhong, Naczk and Shahidi (2008) incorporated protein hydrolysates in a comminuted meat model system and suggested that the use of low molecular weight peptides could be more effective in holding water compared to large-sized peptides while acting as an antioxidant. Onodenalore and Shahidi (1996) and Shahidi et al. (1995) reported similar observations on enhanced cooking yield of comminuted pork with increased addition level of protein hydrolysates from shark and capelin.

The water binding can vary according to both intrinsic and extrinsic factors. Determination of water holding ability is associated with the remaining water in the sample after filter paper press, gravitational filtration or centrifugation (Nakai and Li- Chan, 1988; Cumby et al., 2008). Parameters of water binding capacity could be measured using the difference between the absorbed and retained moisture, swelling properties, considering water vapor of relative humidity and as drip loss volume (Zhang Wang, Xu and Xu, 2019).

Shahidi and Synowiecki (1997) documented a method to determine the suitability of protein hydrolysates (from seal meat) as a water-binding agent and a phosphate alternative to improve the water retention in the muscle food. According to this method, drip volume was calculated as the difference between the initial weight and the final weight and water holding capacity was expressed as a decrease of drip volume against a control (Ambigaipalan and Shahidi, 2015).

2.6.4.4 Fat binding ability

Fat binding capacity plays a vital role in flavor retention, shelf life, emulsification and other sensory attributes of the food (Haque et al., 2016; Deng, Butré and Wierenga 2019). The entrapment of fat into the food matrix is strongly associated with the type of oil and protein involved and mainly the hydrophobicity of the protein (Kristinsson and Rasco, 2000). The surface-related properties of proteins, protein configuration, amino acid composition, protein-protein interactions, the spatial arrangement of the lipid phase, processing conditions and temperature are some of the predominant factors that affect protein-lipid interactions (Wanasundara, 1995). The mechanism of fat binding is not fully understood. However, Zayas (2012) described it based on the physical entrapment of oil-related to the protein material microstructure. Kinsella (1982) also suggested that non-polar side chains of protein molecules are responsible for protein-lipid interactions. Non-covalent interactions, such as hydrophobic and electrostatic bonding, are also crucial for protein-lipid interactions (Wanasundara, 1995). Hydrophobic interactions are mainly involved in stabilizing the interactions of both polar and non-polar lipids (Haque et al., 2016). Electrostatic bonding between protein and lipids include: (a) interaction between negatively

charged phosphate groups of phospholipids and positively charged protein groups (lysyl and guanidyl) and/or (b) positively charged group in phospholipid (e.g., choline) and negatively charged amino acid side chains such as glutamyl, aspartyl (Nakai and Li- Chan, 1988).

Proteins with low solubility and high hydrophobicity may possess strong fat binding capacity (Zayas, 2012). The fat binding ability in protein is well known in the emulsifying function of proteins in food formulations (Haque et al., 2016). In addition, flavor retention behavior associated with fat binding ability is used for designing food flavors (confectionery industry) (Wassawa Tang, Gu and Yuan, 2007). Determination of fat absorption ability of protein is often conducted by mixing excess oil with the protein sample for a specified time (holding time) period followed by a centrifugation step. Fat absorption capacity is expressed as the amount of fat in milliliters that is bound by 1g of protein sample. It is calculated using the difference between the initial amount of added and free oil after centrifugation (Kristinsson and Rasco, 2000).

2.6.4.5 Emulsification

Emulsion is defined as a dispersion of two or more immiscible liquids in which one of the liquid is dispersed in the other. Water-in-oil emulsion or oil-in-water emulsion systems are often used to demonstrate the emulsification activity in a food matrix. Mixtures of immiscible liquids (such as water and vegetable oil) are thermodynamically unstable emulsions (Wanasundara, 1995). The addition of compounds that are partly soluble in both phases may assist the dispersion of the emulsion and stabilize the emulsion system against coalescence. Proteins possess the ability to function as emulsifiers and emulsion stabilizers.

Emulsifying abilities are directly related to the surface properties of proteins (Kristinsson and Rasco, 2000). A complex group of factors of a protein, including amphiphilic nature, interfacial activity, as well as solubility and mobility, determines its emulsifying ability (Lopes-da-Silva and Monteiro, 2019). Interfacial properties are associated with the hydrophobic and hydrophilic moieties. These groups are responsible for the adsorption and stabilization behavior of proteins at air-water and oil-water interfaces (Figure 2.4) (Kristo and Corredig, 2015). The activity of an emulsifier protein depends on its ability to reach the interface of water and lipid and unfolding at the interface.

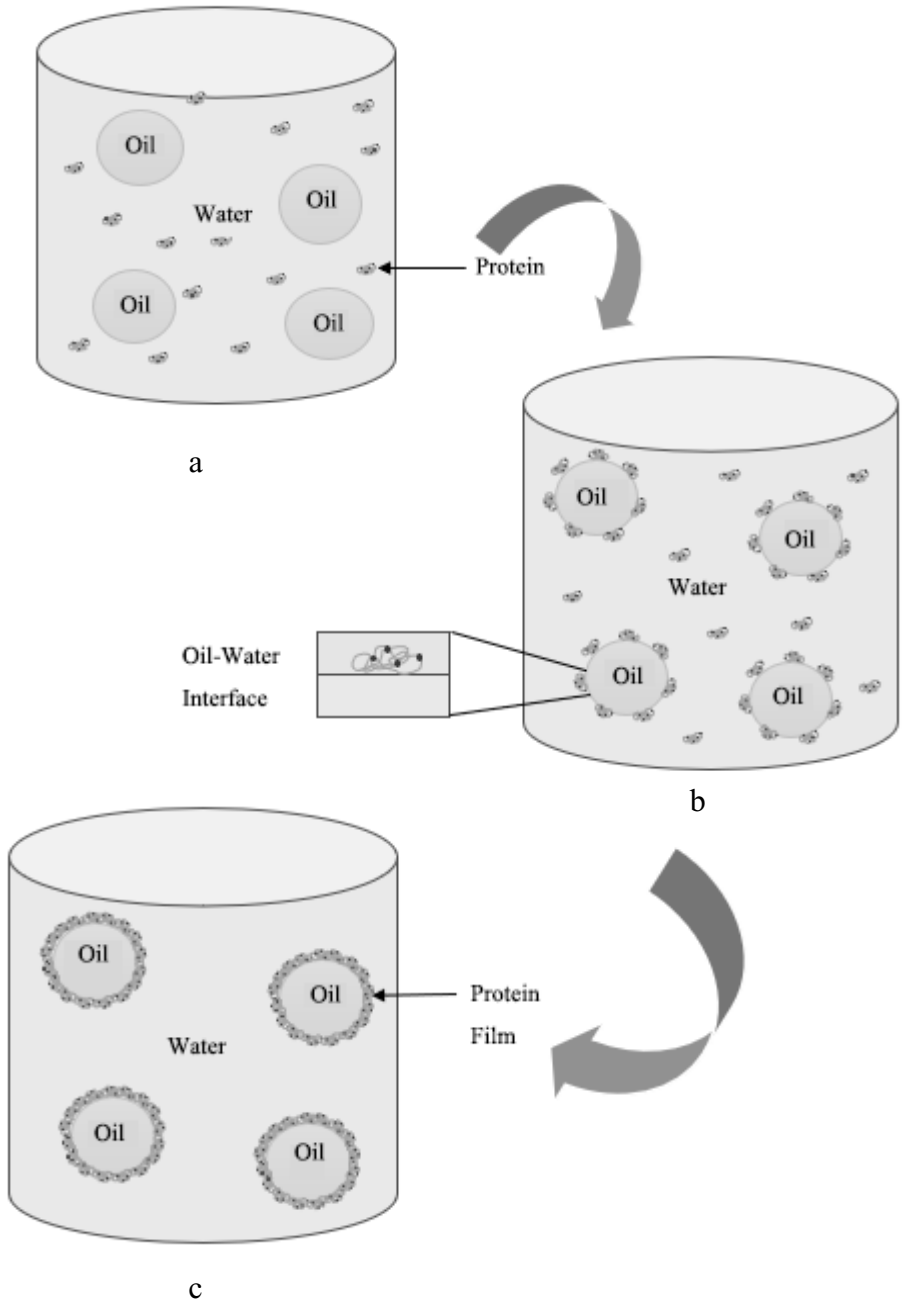


Figure 2.4 Stages of emulsification process of food protein (a: migration of protein to the interface, b: unfolding and rearranging at the interface, c: formation of viscoelastic film)

The unfolding and reorienting at the interface may expose the hydrophobic groups of proteins to interact with the lipid (nonpolar) phase. Thus, key factors for the protein to act as an emulsifier are: (a) the ability of protein molecules to move to the interface (mobility), (b) better solubility to achieve the required mobility, and (c) the ability to unfold at the interface (Lopes-da-Silva and Monteiro, 2019). In addition, Kristinsson and Rasco (2000) stated that peptide length has a positive relation between surface activity and emulsification ability. Peptides should have a minimum length of >20 amino acid residues to exhibit strong emulsifying ability (Kristinsson and Rasco, 2000). The peptide length could become a crucial determinant as the smaller peptides reduce the efficacy of the emulsifying ability. Though the smaller peptides have higher mobility and better adsorption at the oil-water interface, their ability to unfold and rearrange at the interface is weaker compared to larger peptides (Mintah He, Dabbour, Xiang, Agyekum and Ma, 2019). Hence, limited proteolysis is suitable for acquiring a better emulsifying capacity.

Ionic strength and pH are other important extrinsic factors that affect the emulsifying ability of food proteins. Proteins show low solubility and neutral charge at their isoelectric points that directly exerts a negative impact on emulsifying activity (Foegeding and Davis, 2011). In addition, the presence of polysaccharides may improve the emulsifying properties (Lopes-da-Silva and Monteiro, 2019). Interfacial tension also correlates with the emulsification when it starts to deplete with the formation of an emulsion (Wang et al., 2019).

The versatility of emulsifying properties of proteins is effectively applied in various food products. Notably, surface-activity and stabilizing activities of proteins are associated with their film-forming properties. This unique property is adequately used for the

preparation of a wide range of milk products, confectionaries, as well as bakery and meat products (Foegeding and Davis, 2011). The configuration of the protein in the actual food products is related to the emulsion formation that describes the realignment of protein in such a way that hydrophobic groups penetrate the oil droplets while the hydrophilic groups extend into the aqueous phase (Haque et al., 2016). Under the influence of gravity or centrifugal force, the emulsion is allowed to separate into two phases including (a) fat globules compacted into a cream layer (b) the aqueous layer devoid of fat (Wanasundara, 1995). The emulsion activity refers to the ratio between the formed cream layer and volume of the initial emulsion. Emulsion stability describes the change in the phase separation during a specified period at a given temperature and a gravitational field (Panyam and Kilara,1996). Both emulsification activity index (EAI m^2/g) and emulsifying stability index (ESI, min) have been employed to assess the emulsifying properties. In addition, the droplet size distribution in the emulsion is used as an indicator for determining the stability and rheology of the emulsion. It has been reported that higher emulsifying ability is related to smaller droplet sizes (Wu et al., 2019).

2.6.4.6 Foaming capacity

Foams are thermodynamically unstable colloidal systems with a dispersed gas or air droplets in a continuous liquid or aqueous phase (Panyam and Kilara,1996). Therefore, foams are formed by trapping air bubbles in the liquid. Protein film surrounding the air bubble provides the kinetic barrier to the bubble against coalescence and rupture. For an effective foam formation, protein should possess the ability of rapidly migrate, unfold and rearrange at the interface of air-water (Mintah et al., 2019). During the process, protein

solubility and interfacial properties make significant contributions to the foaming behavior of the protein. The amphiphilic nature of the proteins involves decreasing the surface and interfacial tension of the liquid. Reduced surface tension could be achieved through the orientation of unfolding proteins at the interface by extending their hydrophobic moieties into the air and the hydrophilic groups into the aqueous phase (Kristinsson and Rasco, 2000). Panpipat and Chaijan (2017) described the mechanisms associated with the foam coalescence using three possibilities including (a) disproportionation of air bubbles, (b) instability of thin films between the bubbles and (c) dripping of water from the surface of the bubble down to the aqueous layer that may cause the collapse of protein film around the bubble. The use of cohesive multilayer film-forming foam stabilizers could be detrimental to the disproportionation and coalescence of the bubbles (Panpipat and Chaijan 2017).

As previously indicated in the emulsification section, similar molecular properties of proteins are required for foaming. Distinctly, viscoelasticity associated with interfacial properties plays a vital role in preventing the deformation and the coalescence of the droplets at the interface (Foegeding and Davis, 2011). Moreover, improved protein solubility, surface flexibility and increased charge distribution enhance the spread of proteins on the water-air interface resulting in increased absorptivity (Deng et al., 2019). Increasing pH over isoelectric point also contributes to increasing the capacity to encapsulate air during foam formation (Panyam and Kilara,1996). Hence, the pH of the dispersing medium is a determinant factor for acquiring better foamability of food proteins. Improving repulsive intensity and availability of hydrophobic amino acids decrease the

coalescence of air bubbles and leads to stable foam formation (Foegeding and Davis, 2011; Deng et al., 2019).

Foamability in food formulations often used whipping, shaking, sparging or injecting to incorporate air into the protein solution. However, the whipping method is usually used for large-scale industrial production, while for smaller size samples sparging is employed (Wanasundara, 1995). Protein foams are extensively used in the formulation of milk-based products like ice cream, bakery products, fudges, desserts, icing and whipped toppings.

Foam formation is evaluated by referring to foaming capacity and foaming stability of the protein. Foaming capacity is determined by considering the ratio between the volume of the foam formation and the initial volume of the solution while foaming stability is an indicator of stabilized foam formation over a specific time. Shahidi et al. (1995) and Onodenalore and Shahidi (1996) studied the foam formation (whipping ability) and foaming stability of protein hydrolysates instead of using protein. Several other studies have also indicated that controlled or limited hydrolysis of proteins could increase the foam formation. However, it may reduce the foaming stability (Kristinsson and Rasco, 2000). Smaller polypeptides were unable to contribute to healthy film formation around the air bubbles. The viscous, elastic, cohesive, gas-impermeable, and continuous film around air bubbles are required to promote better foam stability (Deng et al., 2019).

2.7 Protein hydrolysates and bioactive peptides

Dietary proteins are essential for the growth and maintenance of the body and serve as sources of energy and essential amino acids (Sarmadi and Ismail, 2010; Shahidi and Li, 2015). Proteolytic modifications of food proteins are regarded as the most efficient methods to improve biological activities. As well as it could enhance the palatability and storage ability of proteins by generating protein hydrolysates and bioactive peptides (Kristinsson and Rasco, 2000).

2.7.1 Protein hydrolysates

Protein hydrolysates are the chemically or enzymatically segmented into peptide fragments of protein (Shahidi, Han and Synowiecki, 1995; Kristinsson and Rasco, 2000). Hydrolyzed protein often exhibits novel functional and health promoting properties beyond their native protein structures (Cumby et al., 2008; Aluko, 2018). Peptides with specific functions can be produced by controlling or customizing the hydrolysis process of proteins. Hence, protein hydrolysis is considered as the primary process for generating peptides with specific amino acid combinations. These peptides possess desired physicochemical properties for numerous applications in the food and pharmaceutical industries (Villamil, Váquiro and Solanilla, 2017).

Hydrolyzed proteins from many animal and plant sources have shown unique bioactivities and multifunctional properties. The most common food protein sources include fish, eggs, milk, oilseeds and pulses (Aluko, 2018). Studies have been conducted on capelin protein hydrolysates (Shahidi et al., 1995, Amarowicz and Shahidi, 1997), seal protein hydrolysates (Shahidi, Synowiecki and Balejko, 1994), egg protein hydrolysates

(You and Wu, 2011), casein protein hydrolysates (Rival, Boeriu and Wichers, 2001), canola protein hydrolysates (Cumby et al., 2008), flaxseed protein hydrolysates (Udenigwe, Lu, Han, Hou and Aluko, 2009), and pea, chickpea and mung bean protein hydrolysates (Aluko, 2008). In addition, numerous investigations were carried out to upgrade the underutilized food processing by-products using the protein hydrolyzation technique. A significant number of studies were conducted on producing protein hydrolysates from marine originated by-products. This includes fish viscera (Villamil et al., 2017), fish scales (Nie, Liu and Liu, 2014), fish frames and bones (Sinthusamran, Idowu, Benjakul, Prodpran, Yesilsu and Kishimura, 2020), fish skin (Kchaou Jridi, Benbettaieb, Debeaufort and Nasri 2020), shrimp by-products (Ambigaipalan and Shahidi, 2017) as well as underexploited marine sources such as sea cucumber (Zhang et al., 2020; Dewi, Patantis, Fawzya, Irianto and Sa'diah, 2020; Lin, Zhu, Zheng, Zhao, Fan and Liu, 2020), sea urchin (Mamelona et al., 2011; Qin et al., 2010) and various other marine sources.

Biological activities of food protein hydrolysates have been extensively reviewed in the literature. The most investigated bioactivities of protein hydrolysates include antioxidant (Shahidi et al., 1995; Amarowicz and Shahidi, 1997), antimicrobial (Najafian and Babji, 2012), antihypertensive (Ambigaipalan and Shahidi, 2017), hypolipidemic (Udenigwe and Aluko, 2012), anti-inflammatory (Nwachukwu and Aluko, 2019), anticancer (Chalamaiah, Yu and Wu, 2018), and immunomodulatory (Bhat, Kumar and Bhat, 2015) properties.

Many studies were also conducted on studying the biological and physicochemical functions in protein hydrolysates produced from sea cucumbers (Table 2.5). Discovered bioactive properties of protein hydrolysates from sea cucumber include; antioxidant (Zhao

et al., 2007; Wang et al., 2010; Mamelona, Saint-Louis and Pelletier, 2010; Liu Chen, Su, and Zeng, 2011; Zhou Wang and Jiang 2012; Abedin et al., 2014; Ghanbari et al.,2015; Yan, Tao and Qin, 2016; Sun et al., 2017); antibacterial (Ghanbari et al., 2012), angiotensin-I-converting enzyme inhibitory (Ghanbari et al.,2015; Han et al., 2017; Zhong et al., 2018; Li et al., 2018), anti-inflammatory (Song et al., 2016), antifatigue (Ye et al., 2017), antiaging (Guo et al., 2020), and anticoagulant (Besharati and Khodabandeh, 2017) activities, among others. Protein hydrolysates from the Atlantic sea cucumber (*Cucumaria frondosa*) were mainly explored for their potential for scavenging free radicals under antioxidant activity (Mamelona et al., 2007; Zhang et al., 2020).

The primary objectives of producing protein hydrolysates are to enhance the nutritional, functional and biological value of the original protein to obtain products with high added value and commercial interest (Villamil et al., 2017). Production of peptides for specific functions by hydrolysis is widely employed in the functional food industry (Chalamaiah, Yu and Wu, 2018). Hydrolysis of food protein liberates active peptides that can provide beneficial physiological activities. Thus, protein hydrolysates diversified the potential uses of native proteins (Udenigwe and Aluko, 2012). Incorporating protein hydrolysates into food formulations is a growing trend due to their multifunctionality and health promoting ability. For instance, hydrolyzed proteins were widely employed in protein supplementary food products as well as used in formulating clinical diets (Lafarga and Hayes, 2017). The inclusion of protein hydrolysates into high-energy supplements (i.e., energy drinks), gastric products, weight-control diets and sports nutrition is a common practice of upgrading protein supplementation products. The clinical use of protein hydrolysates has also become increasingly important for treating various disorders

(Nwachukwu and Aluko, 2019). These clinical uses include treatments for patients with metabolic and digestive disorders, as well as they can be successfully used as solutions for malnutrition, and an alternative to chemotherapy (Clemente, 2000; Udenigwe and Aluko, 2012). In addition to human nutrition, there has been a growing interest in the use of protein hydrolysates in animal nutrition (Hou, Wu, Dai, Wang and Wu 2017).

Table 2.5 Bioactive properties of protein hydrolysates produced from sea cucumber

Bioactive property	Sea cucumber species	Body part of the sea cucumber	Method of producing protein hydrolysates	Identified bioactive compound	Reference
Antioxidant	*Cucumaria frondosa*	Whole sea cucumber	Enzymatic hydrolysis; *In silico* hydrolysis	Low molecular peptides (less than 2kDa)	Zhang et al. (2020)
		Viscera	Enzymatic hydrolysis	Protein hydrolysates	Yan et al. (2016)
		Viscera	Enzymatic hydrolysis	Protein hydrolysates	Mamelona et al. (2009)
	Apostichopus japonicus	Eggs	Enzymatic hydrolysis	Peptides (30 kDa)	Zhang et al. (2017)

	Stichopus vastus	Body wall	Enzymatic hydrolysis	Collagen and gelatin hydrolysates	Abedin et al. (2014)
	Stichopus japonicus	Body wall	Enzymatic hydrolysis	Low molecular peptides (~1.56 KDa)	Zhou et al. (2012)
		Body wall	Alkali extraction and acid precipitation followed by enzymatic hydrolysis	Gelatin hydrolysates	Wang et al. (2009)
	Isostichopus badionotus	Body wall	Gastrointestinal digestion	Low molecular weight peptides	Pérez-Vega et al. (2011)
ACE inhibitory	*Holothuria atra*	Gutted sea cucumber	Enzymatic hydrolysis	Low molecular peptides (less than 3kDa)	Dewi et al. (2020)

Holothuria leucospilota	Gutted sea cucumber	Enzymatic hydrolysis	Low molecular peptides (less than 3kDa)	Dewi et al. (2020)
Bohadschia marmorata,	Gutted sea cucumber	Enzymatic hydrolysis	Low molecular weight peptides (less than 3kDa)	Dewi et al. (2020)
Actinopyga lecanora	Body wall	Enzymatic hydrolysis	Low molecular weight peptides (~0.4kDa)	Li et al. (2018)
	Gutted sea cucumber	Enzymatic hydrolysis	Protein hydrolysates	Sadegh et al. (2016)
Parastichopus californicus	Body wall	Enzyme hydrolysis	Low molecular weight peptides	Liu et al. (2011)

	Isostichopus badionotus	Body wall	Gastrointestinal digestion	Low molecular peptides (less than 3kDa)	Pérez-Vega et al. (2011)
	Acaudina molpadioidea	Body wall	Enzymatic hydrolysis	Low molecular weight peptides (less than 1kDa)	Zhao et al. (2007)
Antiaging	*Stichopus variegates*	Whole sea cucumber	Enzymatic hydrolysis	Low molecular weight peptides (less than 3kDa)	Lin et al. (2020)
Iron-binding ability	*Stichopus japonicus*	Ova	Enzyme hydrolysates	Low molecular weight peptides (0.2-1 kDa)	Sun et al. (2017)

Antifatigue	*Stichopus japonicus*	Whole sea cucumber	Enzymatic hydrolysis	Low molecular weight peptides (less than 1kDa)	Ye et al. (2017)
Anti-proliferative	*Isostichopus badionotus*	Body wall	Gastrointestinal digestion	Low molecular weight peptides (less than 3kDa)	Pérez-Vega et al. (2011)

2.7.2 Bioactive peptides

Biologically active peptides are encrypted in the protein molecules and inactive within their precursor protein (Sarmadi and Ismail, 2010). These peptides are released during the hydrolysis process and exert various bioactivities. In general, bioactive proteins contain 2-20 amino acids (Shahidi and Zhong, 2008), while the composition and sequence of amino acids are crucial to render bioactivity of each peptide (Sarmadi and Ismail, 2010). Some of the bioactive peptides can also be released from the precursor protein during gastrointestinal digestion (Shahidi and Zhong, 2008). However, hydrolysis of protein as a food processing step creates bioactive peptides that are different from naturally occurring peptides (Udenigwe and Aluko, 2012). As indicated in the Table 2.3, many studies have been conducted on body wall of the sea cucumber and reported that partially hydrolysed collagen proteins or gelatin peptides possessed better bioactive potentials (i.e., radical scavenging abilities) than the intact collagen (Zhao et al., 2007; Yan et al., 2018).

The processing approaches, including chemical or enzymatic hydrolysis and bacterial fermentation are used to release the bioactive peptides from a complex mixture with other inactive molecules present (Shahidi and Li, 2015). Recently bioinformatics tools were used to chemically synthesize particular peptide sequences with distinct bioactivities (Udenigwe and Aluko, 2012). In general, most of the hydrolysis processing steps are followed by the post-hydrolysis techniques to isolate the biologically active fractions. The resultant mixture of peptides from food protein hydrolysis is separated into different fractions with distinct amino acid sequences that exert specific functions (Aluko, 2018). Following subsections of the chapter discuss the production methods used for hydrolysing

protein, subsequent steps of purification and isolation processes of bioactive peptides and an overview of bioactive peptide characterization related to antioxidant activity and antihypertensive activity.

2.7.3 Production of protein hydrolysates and bioactive peptides

The choice of a processing method for hydrolyzing the protein of interest depends primarily on the protein source (raw material) and purpose of production (Hou et al., 2017). The most common means of producing protein hydrolysates in the industrial sector include chemical and biological approaches (Kristinsson and Rasco, 2000). In addition, physical modification of food proteins, such as heat treatments, high-pressure applications can also produce potent bioactive peptide mixtures (Wu et al., 2020). The cleavage of peptide bonds in the parent protein leads to liberation of peptide fragments of different sizes and free amino acids. Post-hydrolysis processing techniques are mandatory for identification and isolation of peptides of interest from the resulting complex mixture.

2.7.3.1 Chemical methods

Protein hydrolysis processes employing acids or alkali are generally referred to as chemical hydrolysis methods. These methods are relatively simple and inexpensive, but tend to destroy desirable characteristics of parent proteins (Kristinsson and Rasco, 2000). The lack of selectivity and specificity in hydrolysate products were reported to be high during the chemical hydrolysis (Shahidi and Zhong, 2008; Kim and Wijesekara, 2010).

As consequences of chemical hydrolysis process, most of the functional properties and nutritional qualities of original proteins were adversely affected. Moreover, retention of residual organic solvents or toxic chemicals in the final products limits the use of

chemical hydrolysis of food protein (Kristinsson and Rasco, 2000). For instance, acid hydrolysis deteriorates essential amino acids such as tryptophan, cysteine and cystine and converts asparagine and glutamine into aspartic acid and glutamic acid, respectively. Alkali hydrolysis leads to the elimination of cysteine, lysine, isoleucine, serine and threonine (Kristinsson and Rasco, 2000; Toldrá et al., 2018). However, most of the acid hydrolysis products are used as flavor enhancers, whereas alkaline hydrolysates produce foaming agents (Hou et al., 2017).

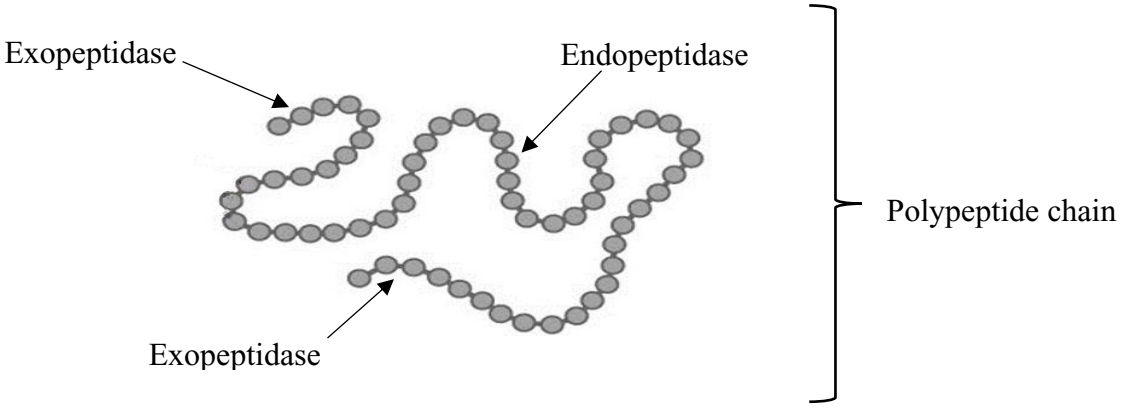
Chemical hydrolysis of food proteins is considered to be harsh and difficult to control. The resultant product might be extensively hydrolyzed and have a deleterious effect on the physicochemical properties in food systems (Kristinsson and Rasco, 2000). Furthermore, both acid and alkaline hydrolysis may produce hydrolytic degradation products through racemization reactions (Kristinsson and Rasco, 2000). Solubility, dispersibility and most of the other quality attributes were affected by the excessive hydrolysis of protein (Clemente, 2000). Therefore, the use of chemical hydrolysis has not been practiced extensively in the food industry (Hou et al., 2017).

2.7.3.2 Biological methods

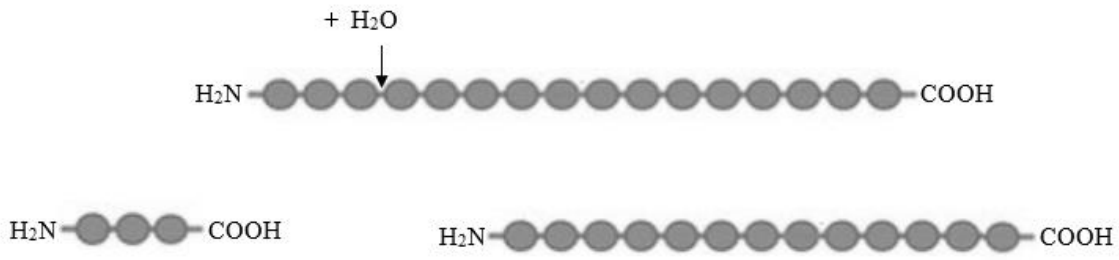
Biochemical approaches to produce protein hydrolysates from food proteins are preferred over chemical methods, mainly due to the enhancement of desirable characteristics associated with the final product and the involvement of mild processing conditions (Shahidi and Zhong, 2008).

Biological methods, typically include(a) enzymatic hydrolysis *in vitro*, (b) bacterial fermentation, (c) gene expression and (d) *in vivo* (or simulated) digestive proteolysis

(Shahidi and Li, 2015). Among these biotechniques, the most employed technique in producing sea cucumber derived bioactive peptide is enzyme-catalyzed proteolysis. Use of enzymatic hydrolysis for the production of biologically active protein hydrolysates associated with various benefits including high rate of recovery of nutritionally and physiologically important protein hydrolysates and applicability to use over wide range of protein sources (Chalamaiah et al., 2012). Enzymatic modifications of proteins are mainly determined by the type of enzyme used in the hydrolysis process. Different types of proteinases, including endo- and exo- proteinases (Figure 2.5) are used to hydrolyze the peptide linkages.



a) Endopeptidase



b) Exopeptidase

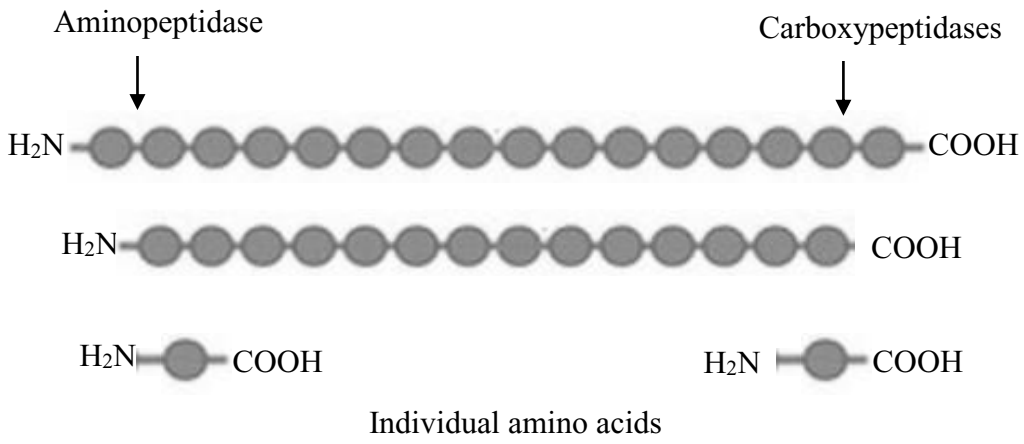


Figure 2.5 Activity of a) endopeptidases and b) exopeptidases on polypeptide chain (Adapted from Ceuleers et al., 2016; https://tcbiologyalevel.wordpress.com/2016/03/31/digestion-and-absorption/)

Endopeptidases hydrolyse the peptide bonds between polypeptide chains, while exopeptidases (aminopeptidase and carboxypeptidase) catalyze the cleavage of the terminal peptide bond from a polypeptide chain or a protein (Panyam and Kilara, 1996; Kristinsson and Rasco, 2000). Furthermore, the hydrolysis activity of exopeptidase could liberate free amino acids from the N- (use of aminopeptidases) and C- terminals of peptides (use of carboxypeptidases) (Toldra et al., 2018)

In addition to protein specificity, other important factors are duration of hydrolysis process, the extent of hydrolysis or degree of hydrolysis, substrate and enzyme concentrations, pH, and pretreatment of the protein before hydrolysis (Shahidi and Li, 2015; Dewi et al., 2020). These parameters have direct impact on the technofunctionality of resultant peptides. Notably, protease specificity determines the molecular weight,

hydrophobic characteristics and functional behaviour of the peptides. Therefore, enzymatic digestion can steer the hydrolysis process to obtain the desirable multifunctional peptides.

Enzymatic hydrolysis can be performed either sequentially or following the zipper mechanism (Panyam and Kilara, 1996). The zipper mechanism refers to the process of hydrolyzing the intermediate hydrolysis products to smaller peptides during the progress of proteolysis (Deng et al., 2018). The mechanism behind the catalytic activity of proteases is based on the nucleophilic attack of a water molecule on the peptide bond between the amino and the carboxylic groups of two adjacent amino acids (Wouters et al., 2016).

Several proteinases of plant, animal and microbial origin are used to hydrolyze food proteins (Najafian and Babji, 2012). Food grade enzymes commonly used in the food industry include Alcalase, Flvourzyme, bromelain, papain, trypsin, chymotrypsin, pancreatin, Neutrase, Orientase, thermolysin and Validase, among others (Amarowicz and Shahidi, 1997; Samaranayaka, and Li-Chan, 2008; Chalamaiah et al., 2012; Udenigwe and Aluko, 2012; Shahidi and Li, 2015; Ambigaipalan and Shahidi, 2017). The choice of enzyme is a crucial factor in producing the final product with predetermined properties. Screening of suitable enzymes is a vital process due to the enzyme specificity (Wouters et al., 2016). In sea cucumber protein research, most of the studies were performed using animal and microbial origin enzymes. Sea cucumber collagen was extracted by hydrolyzing cross-linked molecules using pepsin (animal origin) without degenerating the integrity of the triple helix of the collagen protein (Zhang et al., 2007; Liu et al., 2010; Abedin et al., 2013; Siddiqui et al., 2013; Adibzadeh et al., 2014; Liu et al., 2017).

Proteolytic process could enhance the functionality of protein hydrolysates than the intact proteins (Panyam and Kilara, 1996). Desired functionality is linked with the degree

of hydrolysis and the hydrolysis protocol (Wouters et al., 2016). In general, the degree of hydrolysis is defined as the percentage of peptide bonds cleaved during the hydrolysis process (Adler-Nissen, 1977). Various methods were established for the determination of the degree of hydrolysis. The trinitrobenzenesulfonic acid method (TNBS; Adler-Nissen 1979), the *O* -phthaldialdehyde method (OPA), formol titration method and determining the amount of nitrogen soluble in trichloroacetic acid after hydrolysis are widely used analytical techniques to measure the degree of hydrolysis (Wouters et al., 2016).

In addition to the controlled process of enzymatic hydrolysis as indicated before autolysis or *in vivo* gastrointestinal digestive proteolysis process that is also capable of generating biologically active peptides from the parent protein molecules (Shahidi and Li, 2015). Autolysis basically depends on the activation of digestive enzymes, which is a complex mixture of proteases (Kristinsson and Rasco, 2000). The peptides produced during the autolysis are in different molecular profiles and may possess distinct physiological activities due to their greater bioavailability in target tissues (Shahidi and Li, 2015). Numerous studies have been conducted to investigate the effect of endogenous enzymes of sea cucumber species on collagen fibrils. Endogenous enzymes including cysteine proteinases (Sun et al., 2001; Zhou et al., 2014), serine proteinases (Fu et al., 2005; Yan et al., 2014) and matrix metalloproteinases (Wu et al., 2013; Zhong et al., 2015; Liu et al., 2018) have been characterized from various sea cucumber species. Most of these enzymes are involved in autolysis of sea cucumber. Yan et al. (2014) demonstrated that serine proteinases from sea cucumber could have the ability to cleave the collagen cross-inks.

Nevertheless, several drawbacks associated with the autolysis for the commercial level production of protein hydrolysates are (a) practical difficulties in controlling the

hydrolysis process, (b) unpredictable nature of the produced peptides, (c) species-specific and impact of digestive enzyme concentrations (Kristinsson and Rasco, 2000; Shahidi and Li, 2015). Despite these limitations, the simulated gastrointestinal process has been used to investigate the liberation of bioactive peptides during the ingestion and digestion of dietary proteins (Udenigwe and Aluko, 2012).

Fermentation is another classic biological approach for hydrolyzing food proteins. It is referred as a traditional food processing method. Fermentation of precursor proteins generates protein hydrolysates rich with biologically active peptides (Hartmann and Meisel, 2007). Microbial fermentation of food protein has long been practiced in food formulations. Fermentation of foods such as milk and meat products by employing different bacteria strains could produce potent bioactive peptides (Najafian and Babji, 2012). For example, lactic acid bacterial strains (e.g. *Lactobacillus*) are used to ferment milk proteins during the yogurt and cheese manufacturing process. Protein fermentation is classified into a liquid- or solid-state hydrolysis process. Liquid-state fermentation is carried under high moisture conditions, whereas solid-state fermentation is performed under low-moisture fermentation conditions (Hou et al., 2017).

Most of the hydrolysates produced during the fermentation process are responsible for several bioactivities and organoleptic properties. Peptides present in the fermented products exhibit biological activities, including antioxidant and antihypertensive activities, and contribute to the sensory attributes by generating peptides that impart flavour (Bhat, Kumar and Bhat, 2015). Compared to chemical processing methods, fermentation is an environmentally friendly and energy-saving process (Wu et al., 2020). However, fermentation techniques and the final products are varied according to the type of

fermenting microorganism (Montesano et al., 2020). Moreover, fermented products may contain other biomolecules, including exopolysaccharides, bacteriocins and bacteria cells that could contribute to the biological activities. Therefore, inclusiveness of observed bioactive properties of peptides released during fermentation is another drawback of the microbial hydrolysis process (Agyei et al., 2016). A few limitations associated with the fermentation are (a) less efficiency of the hydrolysis process, (b) inconsistency in the production of peptides, and (c) difficulty of predictable performance of microbial systems (Jin et al., 2016).

All these biological modifications possess unique processing characteristics and create an ecological advantage by improving the nutritional quality and bioaccessibility of protein hydrolysates with minimal influence on the environment (Wu et al., 2020).

2.7.3.3 Physical modification (treatment) methods

Compared to biological and chemical processing of food proteins, physical treatments have increasingly attracted the attention of food processors as an emerging technique to facilitate the generation of protein hydrolysates. Recently, ultrasound treatment, high-pressure applications and microwave treatment are widely used to accelerate the production of food-derived proteins individually or in combinations with biological and chemical methods (Ozuna et al., 2015). Jin et al. (2019) investigated the microwave-assisted enzymatic hydrolysis of collagen from sea cucumber *Acaudina molpadioides*. The findings revealed that produced peptides from collagen fibrils exhibited significant bioactivities, as microwave radiation penetrates the interior of proteins and facilitated the extraction and hydrolysis processes by loosening their structures from the

cell matrix (Jin et al., 2019). In addition, thermal treatments are applied to enhance the enzyme-protein interactions by thermal-induced unfolding of proteins (Udenigwe and Aluko, 2012). Numerous investigations have been conducted to study the efficiency of releasing biologically active peptides from their primary structures of proteins during physical modification process (Wu et al., 2020).

2.7.4 Production of bioactive peptides

According to Udenigwe and Aluko (2012), production of bioactive peptides and strategic selection of food protein source are based on two main objectives: (1) to upgrade protein-rich industrial by-products or improve the value-added use of underutilized proteins, (2) to use protein constituents that are composed of specific peptide sequences with particular pharmacological property. These two approaches can be considered individually or combinedly in the process of choosing the protein of interest and identifying the proteins that can produce predefined peptide sequences.

As described above, protein hydrolysates are a mixture of peptides that can be separated into distinct fractions (Aluko, 2018). The major challenges after hydrolysis of proteins are low purity and low yield of peptides of interest. Thus, post-hydrolysis methods are mandatory to separate the potent bioactive peptides. Most of these post-hydrolysis methods are based on protein purification and isolation principles, which were further expanded to obtain more specific peptides (Figure 2.6) (Agyei et al., 2016).

2.7.4.1 Purification of bioactive peptides

The post-hydrolysis or downstream processing strategies for the primary enzymatic hydrolysates of food proteins are generally selected based on the interested

physicochemical and structural properties of peptides. The physicochemical properties of peptides are decided according to their hydrophobicity, net charge, and molecular weight of the peptides (Shahidi and Li, 2015). Appropriate additional separation methods are employed for further purification of peptides. Membrane ultrafiltration, chromatographic methods and selective precipitation techniques are widely used to accumulate the peptides of defined molecular weight range (Udenigwe and Aluko, 2012; Agyei et al., 2016).

Moreover, reversed-phase HPLC can be employed to separate the peptides based on their hydrophobic nature (Pownall, Udenigwe and Aluko, 2010). Peptide fractions with net charges can be acquired by selective ion exchange chromatography while adsorption chromatography is used to enrich specific amino acids of interest in peptide mixtures. For example, a peptide fraction with fewer aromatic amino acids can be obtained after passing of the hydrolysates through a column packed with activated carbon or by mixing with activated carbon (Udenigwe and Aluko, 2010; Shahidi and Li, 2015). In addition, several research advances have been made to achieve better separation efficiency of bioactive peptides. Among them, electrodialysis-ultrafiltration (EDUF) membrane technology has gained much attention due to the high efficiency of separating low molecular weight peptides with a net charge (Firdaous et al., 2009).

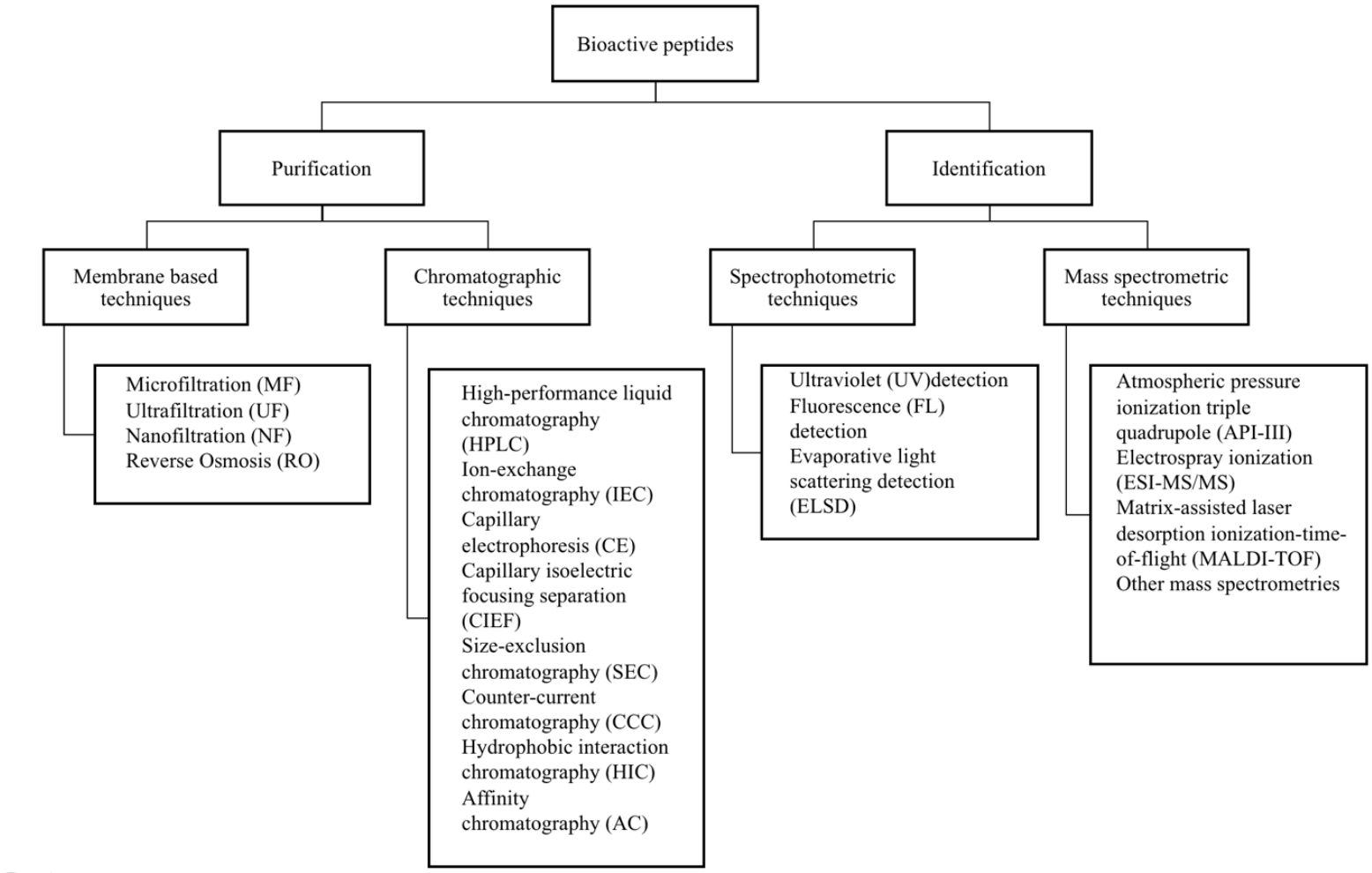


Figure 2.6 Major techniques for purification and identification of bioactive peptides (Adapted from Shahidi and Zhong, 2008)

2.7.4.2 Identification of bioactive peptides

The classical approach of identification and processing of bioactive peptides involves *in vitro* protein digestion followed by the chromatographic purification of hydrolysates. The enrichment of the fractions often performed using ultrafiltration techniques. Separated fractions were then analyzed for the biological activities to identify the bioactive fraction(s). The characterization of the peptides is mostly based on combinations of different methods, including high-performance liquid chromatography and mass spectrometry. The confirmation of the bioactivity is usually performed by chemically synthesizing the peptide sequences and conducting bioactivity testing (Agyei et al., 2016). However, this classical approach has its own merits and demerits. The major drawbacks of the classical approach include: (a) low yields of isolated peptides, (b) limited sample scope, (c) time consumption with laborious nature of the down-stream purification process and (d) possibility of compromising some of the bioactive peptides during the process (likelihood of losing synergistic effects of other components towards the bioactive property), among others (Udenigwe, 2014).

The exploitation of novel alternative purification and fractionation approaches is important in the commercialization of bioactive peptides (Agyei et al., 2016). Application of a computer-based bioinformatic approach (often referred to as *in silico* methods) to generate bioactive peptides has been identified as an effective method in peptide research. The *in silico* approach is based on the information available on the databases with the possible predictions of amino acid sequences that exhibit potent bioactivities. The *in silico* methods are performed when prior knowledge of structure-function properties of active sequences are available. Moreover, detailed experiments should be conducted to confirm

the bioactivity of predicted peptide sequences (Udenigwe and Aluko, 2012). In addition to *in silico* analysis, '-omics' techniques (such as peptidomics, foodomics) are employed to predict and validate the biological and physicochemical properties of food-derived peptides (Agyei et al., 2016).

2.7.4.3 Bioinformatic approach of producing bioactive peptides

Use of '-omics' techniques could replace the existing 'trial and error approach' in peptide purification and activity research. The downstream purification process of bioactive peptides can be accelerated by the integration of *in silico* methods with '-omics' approach. The bioinformatic tools, including universal protein and peptide data bases (UniProtKB, SwissProt, BIOPEP and PepBank) are employed to obtain high throughput related to *in silico* protein digestion and peptide prediction information on potent bioactive peptide sequences (Udenigwe, 2014). These computational tools assist in studying and validating the biological, chemical and physicochemical properties of bioactive peptides before performing their wet-lab synthesis (Agyei et al., 2016). However, the major setbacks of the bioinformatic approaches include: (a) the difficulty of experimentally reproducing the exact predicted *in silico* peptides (b) the possibility of missing the opportunities of novel peptide discoveries as these bioinformatic tools consider only the sequences that currently exist in peptide databases (Udenigwe, 2014). Figure 2.7 summarises the classical and bioinformatics approaches towards the discovery of bioactive peptides from food proteins.

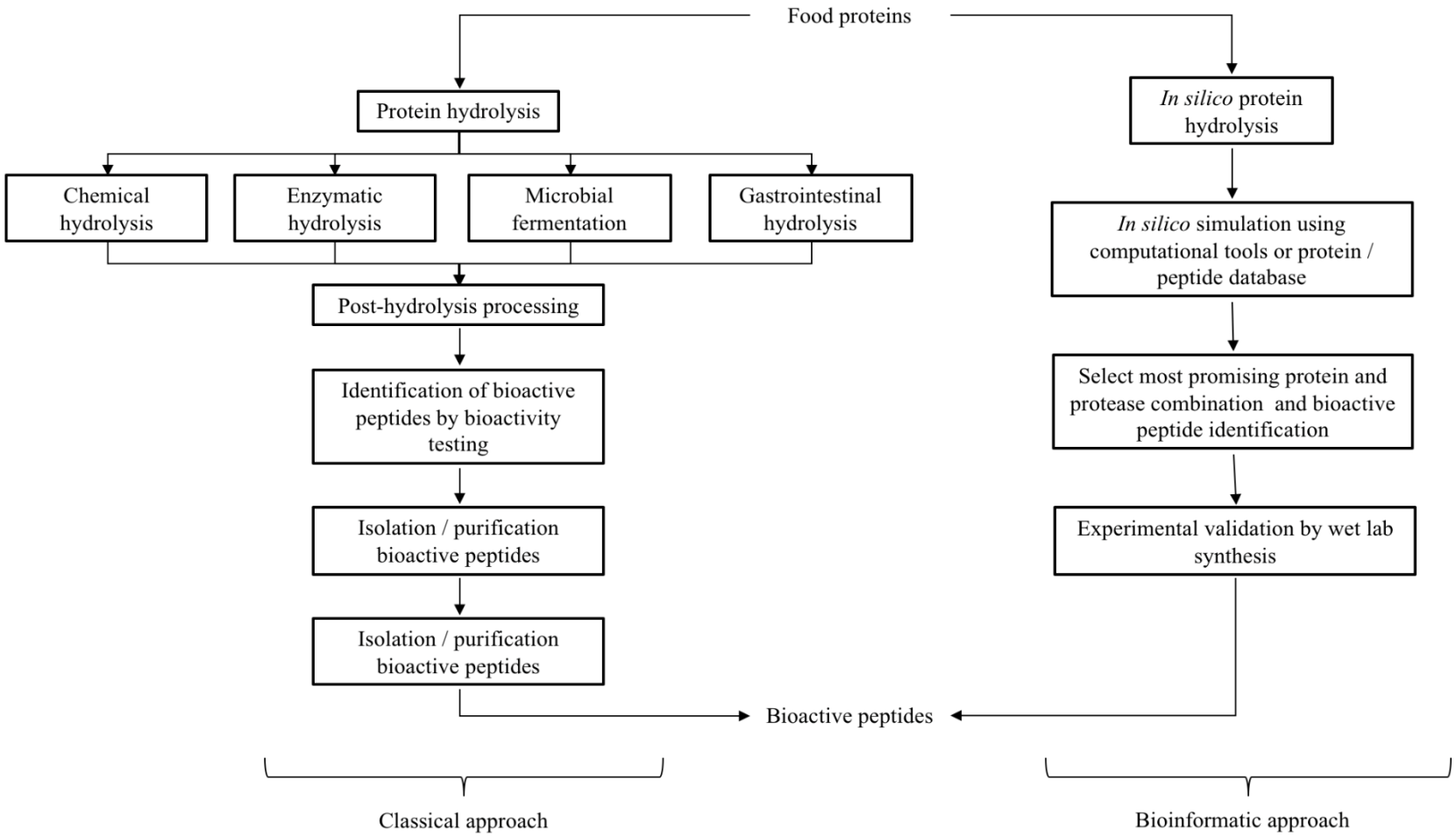


Figure 2.7 Production of bioactive peptides (Adapted from Udenigwe, 2014)

2.7.5 Characteristics of bioactive peptides

As explained in section 2.6.4, bioactive peptides are known to render the different biological activities and exhibit various physicochemical properties. In partially hydrolyzed protein products, most of the structural properties of proteins could influence the physicochemical characteristics of the resultant peptides (Agyei et al., 2016). The multifunctional characteristics of bioactive peptides demonstrate a wide range of applications as described earlier in this chapter.

2.7.5.1 Physicochemical properties

Hydrolysis of food proteins causes the intra- and inter-molecular rearrangement and conformational changes in protein molecules. Unfolding of protein and releasing of encrypted peptides have usually displayed completely different properties in the resulting peptides. Physicochemical properties including amino acid sequence, molecular weight, charge distribution, acid/base group ionization, hydrophilicity/ hydrophobicity indices of peptides could be affected by the hydrolysis process (Cumby et al., 2008; Pownall et al.,2010; Shahidi and Li, 2015; Agyei et al., 2016). Changes in functional properties of native proteins are linked with the peptides and free amino acids produced during the hydrolysis. Notably, hydrodynamic properties such as solubility could be improved over a wide range of ionic strengths and pH levels (Panyam and Kilara, 1996; Kristinsson and Rasco, 2000). Solubility can significantly affect most of other functional properties of the proteins (Benjakul et al., 2014). The mechanism behind enhanced solubility of protein hydrolysates is the increased exposure of more charged and polar groups to surrounding

water after hydrolysis. The hydrophobic groups of proteins convert to hydrophilic groups by liberating small soluble peptides and ionizable functional groups (i.e., carboxyl, amine groups) during the hydrolysis process (Halim, Yusof and Sarbon, 2016). A recent study on extraction and characterization of collagen from sea cucumber (*Holothuria cinerascens*) revealed that resultant product of pepsin assisted extraction is rich in polar groups, including carboxyl, hydroxyl and guanamine groups. These groups are capable of forming a hydrogen bond with water and allow collagen hydrolysate to interact with water (Li et al.,2020). Variation of functional characteristics such as solubility can be attributed to: (a) the net charge of peptides that changes as pH moves away from isoelectric points (pI); (b) the surface hydrophobicity that promotes aggregation through hydrophobic interactions (Halim et al.,2016).

Furthermore, water holding capacity, texture, emulsification properties, foaming properties and organoleptic properties are also improved by the proteolytic digestion of the precursor protein. Number of studies demonstrated that sea cucumber protein hydrolysates possess significant physicochemical properties. Among them, pepsin solubilized collagen and its hydrolysates from sea cucumber (*Holothuria cinerascens*) exhibited high moisture retention capacity compared to pepsin solubilized collagen from tilapia and porcine skin (Li et al.,2020) whereas protein hydrolysates prepared from sea cucumber (*Stichopus japonicus*) guts showed higher whiteness and better functional properties (solubility, water/ oil holding capacity, foaming capacity and emulsification capacity) compared to its untreated counterparts (Du et al., 2019). These enhanced properties lead to expand the utilization of multifunctional peptides in the contemporary industries including food, pharmaceutical as well as cosmetics (Agyei, Potumarthi and Danquah, 2015).

2.7.5.2 Biological properties of bioactive peptides

Biologically active peptides can induce desirable physiological responses upon entry and absorption into the body (Agyei et al., 2016). Depending on the amino acid sequence and composition, bioactive peptides have demonstrated various physiological functions such as antioxidant, antihypertension, immunomodulatory, antithrombotic, opioid, anti-cancer, and antimicrobial activities, among others. The specific bioactivity of peptides is predominantly determined by the structural properties of the amino acid residues. These structural properties, such as chain length of the bioactive peptide, could vary from di-, tri-, and oligopeptides to high molecular weight polypeptides (Shahidi and Zhong, 2008; Udenigwe, 2014). The use of dietary bioactive peptides has exhibited significant effect on disease risk reduction and shows important advantages with respect to elemental diets (Clemente, 2000). Thus, great interest has been given to the application of dietary peptides in clinical diets and their substantial evidence for potential health claims. However, the structure and functional relationship of biologically active peptides is still not well defined.

Most studies are dedicated to identifying food protein-derived bioactive peptides that have the potential to regulate body functions. As indicated previously (in the Table 2.5), sea cucumber derived peptides have been exhibited wide array of bioactivities with beneficial characteristics. Protein absorption is efficient in the form of short-chain peptides due to the availability of peptide-specific cellular sites as well as their ability to enter the peripheral blood circular system and exert their physiological functions (Shahidi and Zhong, 2008). Physicochemical properties of peptides such as molecular size, charge, lipophilicity and solubility could influence their bioavailability in the target tissues. Intact

absorption of peptides occurs through different mechanisms, including paracellular route, passive diffusion, transportation via carriers, endocytosis and lymphatic system (Sarmadi and Ismail, 2010). For example, oligopeptides are absorbed through passive transport, whereas peptides consisting of 2-6 amino acids are absorbed more efficiently than proteins and free amino acids of a similar size (Sarmadi and Ismail, 2010; Udenigwe and Aluko, 2012). In addition to the chain length, the presence of certain amino acids could interfere with the bioavailability. It has been reported that the presence of proline and hydroxyl proline showed resistance to digestive enzymes (Benjakul et al., 2014). Furthermore, smaller size peptides have demonstrated resistance to peptidolysis and are directly absorbed into blood circulation (Udenigwe and Aluko, 2012). Therefore, *in vivo* bioactivity of peptides might not be the same as *in vitro* activity. The *in vivo* bioactivity of peptides may be lower than expected due to the absorption, bioavailability and susceptibility to degradation. On the contrary, it has been suggested that *in vivo* bioactivity of peptides can be higher than *in vitro* activity because of additional gastrointestinal protease activity on these peptides (Sarmadi and Ismail, 2010). Nevertheless, the exact mechanism underlying the difference between the *in vivo* and *in vitro* forms of the bioactivities of peptides is not fully understood.

According to targeted pharmacological uses, the bioactivity of peptides is primarily determined by the constituent amino acid residues and the sequences of particular peptides (Shahidi and Zhong, 2008). The following subsections (2.7.6.2.1 and 2.7.6.2.2) are further discussed the structure and function relationships associated with the antioxidant activity and antihypertensive activity of bioactive peptides.

2.7.5.2.1 Antioxidant peptides

Bioactive peptides have been extensively investigated for their significant antioxidant activities in health promotion as well as in food preservation. In particular, antioxidant peptides are actively involved in the removal of reactive oxygen species (ROS) and responsible for oxidative stress. Oxidative stress occurs when the endogenous defense system fails to prevent the excessive production of reactive radicals (Ambigaipalan and Shahidi, 2017). The imbalance of the oxidative process can result in cellular damage, which further initiates a series of chronic diseases such as cancer, atherosclerosis, diabetes, aging and many other physiological disorders. In addition, oxidation in fats and oil during processing and storage in food products is another detrimental outcome of the oxidative process. Consequently, the nutritional quality of the food product could be deteriorated whilst the organoleptic attributes could be adversely affected (Sarmadi and Ismail, 2010).

The mechanisms of action of antioxidant activities of peptides are exhibited by scavenging or quenching of ROS/free radicals, chelation of transition metal ions, ferric reducing power and inhibition of ROS-induced oxidation of biological macromolecules such as lipids, proteins and DNA (Shahidi and Li, 2015). The ability of peptidic amino acid residues to donate hydrogen atoms (hydrogen atom transfer -HAT) or transfer electrons (Single electron transfer reaction -ET) to free radicals under physiological pH conditions causes the radical quenching activities of food-derived peptides (Udenigwe and Aluko, 2012). The nature of the ROS and reaction medium are the main determinants of the antioxidant properties of individual amino acid residues.

The aromatic amino acids including phenylalanine, tyrosine, tryptophan and nucleophilic sulfur-containing amino acid such as cysteine and methionine as well as other

amino acid residues such as histidine and proline have been reported to exhibit strong antioxidant activities in food and biological systems (Sarmadi and Ismail, 2010). The structure-function studies on bioactive peptides revealed that the imidazole group of histidine involved in both ET and HAT reactions. The antioxidant activity of histidine may be attributed to the chelating and radical-trapping ability of the imidazole ring as well as the quenching of active oxygen and scavenging of hydroxyl radicals (Sila and Bougatef, 2016). Carnosine is a histidine-containing dipeptide derived from muscle cell which displays significant antioxidant activity in a biological system. Furthermore, tripeptide unit Proline-Histidine-Histidine sequence exhibited highest antioxidant activity over 28 tested peptides (Chen et al., 1998). In addition, removal of histidine residue from C-terminal can reduce the antioxidant activity while the addition of hydrophobic amino acids like proline and leucine to the N-terminus of His-His dipeptide could enhance the antioxidant activity of peptides (Udenigwe and Aluko, 2012; Shahidi and Li, 2015). The hydrophobicity of peptides increases the accessibility to hydrophobic cellular targets like polyunsaturated fatty acid chains on the biological membrane, thus enhancing the antioxidative properties of peptides. Furthermore, peptides with strong antioxidant activities include phenylalanine, tyrosine, tryptophan residues that possess electron dense aromatic rings which can chelate prooxidant metal ions. Phenylalanine can form more stable hydroxylated derivatives (para-, meta- or ortho- substituted) by scavenging hydroxyl radicals (Shahidi and Li, 2015). Dipeptides with tyrosine and tryptophan at the amino terminus and histidine and methionine at the carboxyl terminus showed potent antioxidant activity in an aqueous system (Sila and Bougatef, 2016). Studies have also shown that the sulfhydryl (-SH) group contributes to the antioxidant potential in cysteine by directly interacting with radicals

(Udenigwe and Aluko, 2012). Apart from amino acid composition, the molecular weight of peptides also crucial in determining the potent antioxidant peptides. Peptides of molecular weight 500- 1500 Da have shown more significant antioxidant activity than those with a molecular weight above 1500 Da or below 500 Da (Samaranayaka and Li-Chan, 2011).

Moreover, the structural configuration of peptides and the presence of specific amino acids in the peptide have a significant effect on the overall antioxidant activity. For example, the substitution of D-histidine in the place of L-histidine displayed reduced antioxidant activity. It has been shown that the correct positioning of imidazole group is influential for overall antioxidant activity (Shahidi and Li, 2015). Apart from structural properties, certain processing parameters can also impact on the biological activity of peptides. These include the degree of hydrolysis, type of protease, operational conditions, peptide structure and peptide concentration, among others (Sarmadi and Ismail, 2010).

Determination of the antioxidant potential of peptides is based on variety of mechanisms. The activity can be monitored by different measurement assays, including ET, HAT, reducing power and metal chelation assays, among others (Shahidi and Zhong, 2015). Depending on the chemical reactions involved, most of these assays require a chemical system consisting of an oxidant (free radical or other ROS), an oxidizable probe (if necessary) and antioxidant which is intended to be tested. The antioxidant potential of the tested material is usually expressed as an inhibition against ROS-mediated oxidation of the probe or equivalents of a selected standard antioxidant, including trolox and ascorbic acid or other reference materials (Ambigaipalan and Shahidi, 2017). The trolox equivalent capacity (TEAC), 2,2-diphenyl-1-1-picrylhydrazyl radical scavenging capacity (DPPH)

and ferric ion reducing antioxidant power (FRAP) are considered as ET-based assays. The HAT-based assays include oxygen radical absorbance capacity (ORAC) and the β-carotene bleaching assay, among others (Sarmadi and Ismail, 2010). The major drawback of these chemical assays is *in vitro* measurements could not completely represent the antioxidant capacity under *in vivo* conditions (Shahidi and Li, 2015). To overcome some of these limitations, antioxidant efficacy was determined using model systems. Measurement of antioxidant potential in biological model systems and food model systems are used to evaluate the behavior and effectiveness of antioxidants of interest. LDL-cholesterol oxidation inhibition assay, inhibition of DNA oxidation and nicking and cellular assays are some examples of biological model system assays. Similar to biological model systems, food model systems also used oxidation initiators, oxidation markers and different substrates and reaction conditions to evaluate the antioxidant potential of peptides. Most of the antioxidant activity tests in food model systems are performed in model emulsion systems (oil-in-water emulsion, water-in-oil emulsion system) and muscle food model systems, which are prone to oxidation. Moreover, measurements of oxygen consumption, change in oxidation substrate and oxidation products, are also monitored for evaluating a suitable antioxidant agent (Shahidi and Zhong, 2015). Therefore, proper method selection and a combination of assays are recommended for a valid assessment of the antioxidant potential of bioactive peptides.

Recent research interests have been focused on finding effective antioxidants that originate from natural sources. As an alternative to those from plant origin, marine sources of antioxidants exhibit excellent protection against oxidative stress in the body and lead to disease risk reduction (Shahidi and Ambigaipalan, 2015). Sea cucumbers exert strong

antioxidant activity by scavenging reactive oxygen species (ROS) (Xu et al., 2018). Notably, peptides from sea cucumber hydrolysates were also found to possess antioxidant activities mainly due to their molecular weight, amino acid composition and hydrophobicity (Zou et al., 2016; Xu, Zhang and Wen, 2018). For example, enzymatically prepared hydrolysates of *Actinopyga lecanora* possessed strong antioxidant potential as reflected in their ability to scavenge DPPH radical and chelate ferrous ion (Ghanbari et al., 2015). Antioxidant activities of *Stichopus japonicas* peptides were evaluated using hydroxyl radical and superoxide radical anion scavenging activities (Wang et al., 2009; Zhou et al., 2012). Jin et al. (2019) characterized antioxidant peptides produced by microwave-assisted enzymatic hydrolysis of collagen from sea cucumber *Acaudina molpadioides*. The antioxidant activity of these peptides was associated with the low-molecular-weight and presence of hydrophobic amino acid residues (Jin et al., 2019).

2.7.5.2.2 Antihypertensive peptides

Hypertension is one of the leading risk factors for cardiovascular diseases. Blood pressure is physiologically regulated by the renin-angiotensin system (RAS) and kinin-nitric oxide (NO) system (Udenigwe and Aluko, 2012). The RAS is the central targeted regulatory system to treat hypertension in which angiotensin-I-converting enzyme (ACE) plays a vital role. ACE is a membrane-anchored dipeptidyl peptidase that hydrolyzes angiotensin I (AT-I) to the potent vasoconstrictor angiotensin II (AT-II) and also catalyzes the inactivation of bradykinin, a vasodilator. The overall effect of ACE leads to elevated blood pressure by the activation of vasoconstriction and prevention of vasodilation. Thus, inhibitors of ACE have been identified as effective antihypertensive agents. Clinical studies

of ACE inhibitors revealed the significance of using ACE inhibitors to treat patients with myocardial infarction or heart failure and prevent hypertension (Toldra et al., 2018).

Food-derived peptides have extensively investigated as potent ACE inhibitors for their potential use as natural alternatives to the synthetic antihypertensive agents such as captopril, lisinopril and enalapril (Elavarasan et al., 2016). The mechanism of ACE inhibition by food protein-derived peptides has been characterized by the competition of peptides with ACE substrate for the catalytic sites of enzymes. However, some peptides also exhibited a non-competitive mode of inhibition for ACE substrates. It has been reported that peptic ACE inhibitors have consisted of short amino acid sequences with a high concentration of hydrophobic amino acids. Notably, active dipeptides or tripeptides for ACE inhibition includes hydrophobic amino acid residues such as tryptophan, tyrosine, proline and phenylalanine at the C-terminus and aliphatic amino acids such as leucine, valine and isoleucine at the N-terminus of the peptides (Lafarga, and Hayes, 2017). In addition, positively charged amino acids such as lysine and arginine were suggested for the position adjacent to the C-terminus of the peptide whilst residues consisted of bulk side chains, and hydrophobic side chains with high electronic properties may further enhance the ACE inhibitory activity of peptides (Udenigwe and Aluko, 2012).

ACE inhibitory peptides display weak solubility and poor dissolution rates due to the high proportion of hydrophobic amino acids present in their sequences. Therefore, ACE inhibitory peptides are required to have high doses to achieve therapeutic effect during oral administration process (Udenigwe and Aluko, 2012). Moreover, these bioactive peptides should preserve the intact form until they reach to the target sites to exert the desired biological activity. Hence, these peptides should be resistant to degradation of digestive

enzymes, acids and plasma peptidases under the physiological conditions. Therefore, protective measures including chemical modifications and encapsulation strategies can be employed to preserve the intact form of peptides and their specific motifs against gastric degradation (Onuh and Aluko, 2019). These bioactive peptides can be transported as intact peptides across the epithelial cells in the small intestine via a paracellular route or through specific transporters (Lafarga and Hayes, 2017). The inhibitory potential of bioactive peptides against ACE is assessed using *in vitro* and *in vivo* analytical methods mainly focusing on achieving substantial blood pressure lowering effects (Udenigwe and Aluko, 2012).

Recently, several studies have indicated the potential antihypertensive activity of different sea cucumbers. Abedin et al. (2015) identified ACE I inhibitory peptides from the trypsin hydrolysates produced from the sea cucumber (*Stichopus vastus*). Under optimum conditions, ACE inhibitory activity was around 67.8% and presence of proline, phenylalanine and tyrosine at the C-terminal peptide sequence mostly determined the ACE inhibitory activity of bioactive peptides recovered from the hydrolysates of sea cucumber (*Stichopus vastus*) (Abedin et al., 2015).

Protein hydrolysates from sea cucumber (*Actinopyga lecanora*) were produced using Alcalase, papain, bromelain, Flavourzyme, pepsin, and trypsin and the ACE inhibitory activity was evaluated (Ghanbari et al., 2015). Hydrolysates prepared with Alcalase exhibited the highest ACE inhibitory activity (69.8%) after 8 h of hydrolysis. The ACE inhibitory activity of protein hydrolysates was found to improve with the extended hydrolysis time up to 8 h (Ghanbari et al., 2015). In another study, ACE inhibitory activity

was investigated for the peptides released by the *in vitro* simulated gastrointestinal digestion of sea cucumber *Isostichopus badionotus* (Pérez-Vega et al., 2013).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Fresh sea cucumbers (*Cucumaria frondosa*) were harvested from Northwest and Southeast regions of the St. Pierre Bank (NAFO Division 3Ps), Newfoundland, Canada. Alcalase (2.4 AU/ g) and Flavourzyme (1000 LAPU/g) were purchased from Novozymes, Bagsvaerd, Denmark. Corolase 7089 was procured from AB enzymes GmbH, Darmstadt, Germany. Angiotensin I-converting enzyme (ACE) from rabbit lung, ACE synthetic substrate hippuryl-L-histidyl-L-leucine (HHL), pBR 322 plasmid DNA, and SYBR safe DNA gel stain were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). All chemicals and solvents used were of analytical or chromatographic grade and obtained from Sigma-Aldrich Canada Ltd. (Oakvillie, ON, Canada) or Fisher Scientific Ltd. (Ottawa, ON, Canada).

3.2 Methods

Figure 3.1 summarizes the overall experimental design of the study.

3.2.1 Preparation of protein isolates

Fresh sea cucumbers were dissected and separated into body wall (BW), flower (FL), and internal organs (IN). Tissues of separated body parts were freeze-dried using a Labconco FreeZone benchtop freeze dryer (Labconco, Kansas City, MO) for 72 h to remove the moisture (Figure 3.2). Dried samples were ground to particle size ≤ 100 microns. The fine powder so obtained was then defatted according to Ambigaipalan and

Shahidi (2017). Ground sea cucumber samples were blended with hexane (1:5, w/v) for 5 min in a Waring blender (model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT, USA) at room temperature. The procedure was repeated three times and defatted samples were vacuum-packed and stored at -20 °C for further analysis.

Freeze dried powders of each tissue were separately dispersed in distilled water (10%, w/v). The dispersion was adjusted to pH 11.0 ± 0.5 with 1M NaOH. Alkaline pH values were selected according to preliminary experiments (data not shown). The dispersions were stirred for 1h using a magnetic stirrer followed by centrifugation (10,000 x g) for 20 min in a refrigerated centrifuge (Thermo Scientific™ Sorvall LYNX 6000 Superspeed Centrifuge, Thermo Fisher Scientific, Pittsburgh, PA, USA). The mixture was separated into two distinct layers - alkaline-solubilized protein in the top and insoluble material in the bottom layer. The top layer (supernatant) was collected, with the residue being re-extracted as described above. Supernatants containing protein fractions were combined and adjusted to pH 5.5±0.5 with 1M HCl. Isoelectric pH for each sample was identified in preliminary experiments. Once the desired pH was obtained, the mixtures were stirred for an additional hour and the precipitates were recovered after centrifugation at 10,000 g for 20 min. Subsequently, the pH was adjusted to neutral, and the samples were dialyzed and freeze-dried to obtain body wall protein isolate (BWI), flower protein isolate (FLI), and internal organ protein isolate (INI). Freeze dried protein isolates were stored at -20°C for further analysis.

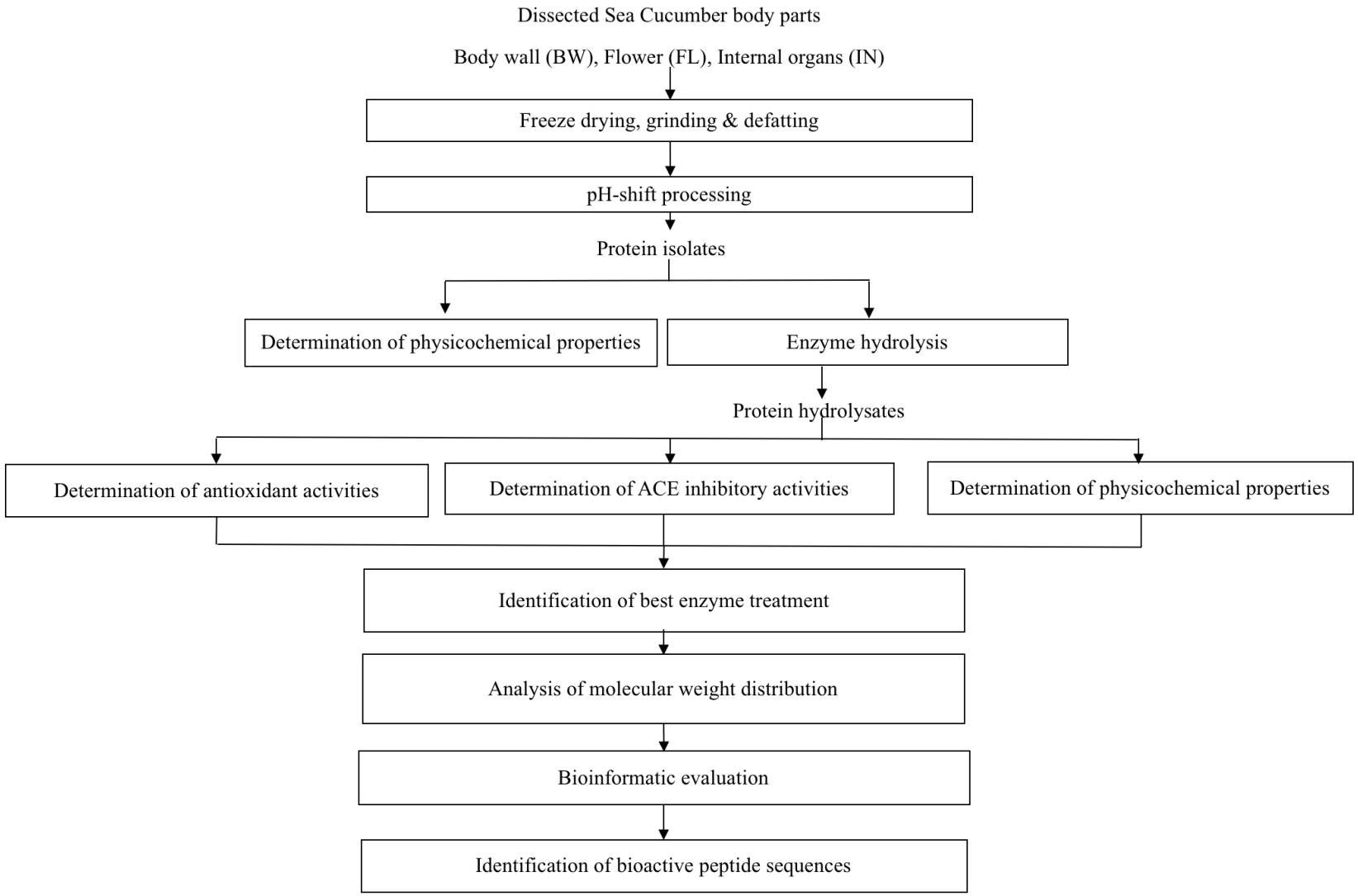
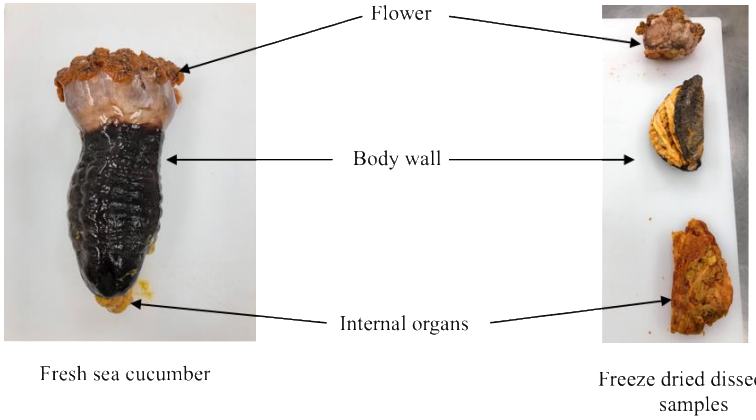


Figure 3.1 Experimental design for evaluation of protein isolates, hydrolysates and bioactive peptides derived from sea cucumber



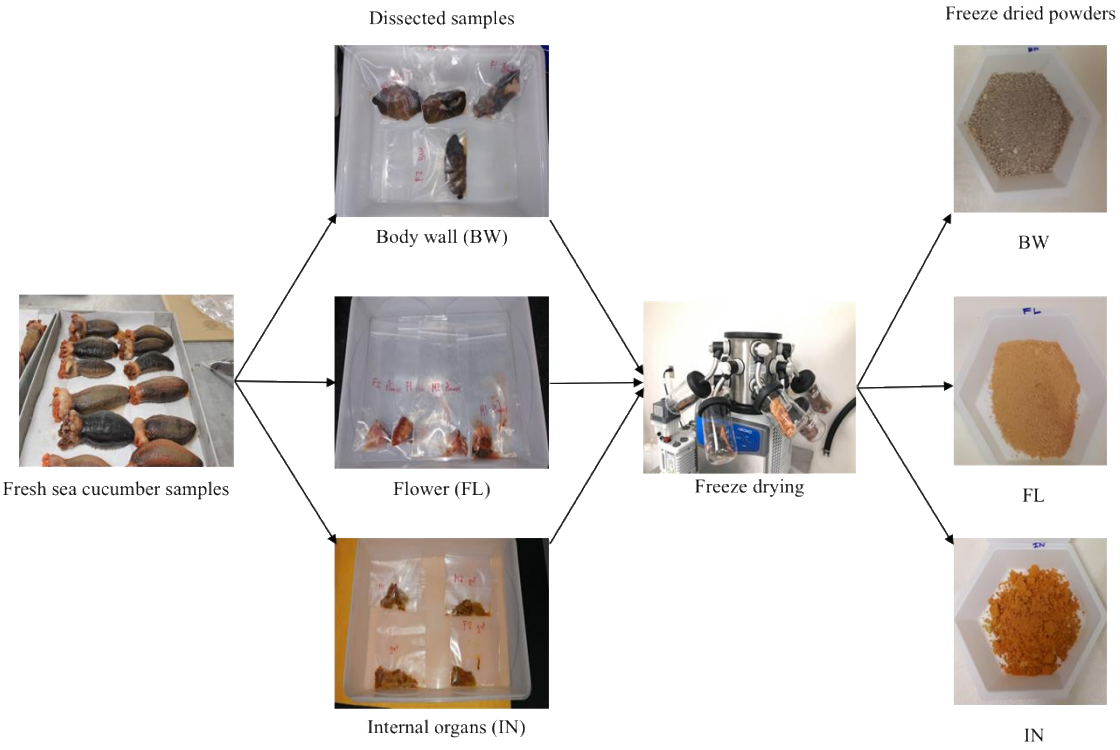


Figure 3.2 Sea cucumber sample preparation

3.2.2 Physicochemical properties of protein isolates

3.2.2.1 Amino acid composition

The amino acid composition of each protein isolate was analysed at the Analytics, Robotics and Chemical Biology Centre (SPARC BioCentre), The Hospital for Sick Children, Toronto, ON, Canada, as reported by Mohan and Udenigwe (2015). Except for tryptophan, cysteine and methionine analysis, all the other amino acids were analysed using vapour-phase hydrolysis with 6 M HCl, 1% phenol at 110 °C for 24 h. For tryptophan analysis, samples were hydrolysed with 4.2 M NaOH for 24 h at 110 °C. Cysteine quantification was carried out by performic acid oxidation prior to hydrolysis. Norleucine (25μM/mL) was used as internal standard for all samples. Following hydrolysis, samples were dried and resuspended in a redrying solution of methanol/ water/ triethylamine (2:2:1, v/v/v) and vacuum dried for 15 min. This was followed by pre-column derivatization with a derivatizing solution made up of methanol/ water: /triethylamine/ phenyl isothiocyanate (PITC) (7:1:1:1, v/v/v). The derivatized samples were vacuum dried and dissolved in sample diluent. Diluent aliquots were then analysed by a high-performance liquid chromatography system, Waters ACQUITY UPLC (Milford, MA, USA) consisting of a 10-cm C-18 column (0.21×10 cm) running on a modified PICO-TAG gradient at 48°C. The amino acids were quantified based on the peaks monitored at 254 nm with a UV detector.

3.2.2.2 Color measurement of protein isolates

The color of the freeze-dried hydrolysates was measured by a Hunter lab color meter (Hunter Associates Laboratory Inc, Reston, VA, USA) and reported using CIE system, where L, a and b parameters indicate lightness, redness, and yellowness, respectively.

3.2.2.3 Solubility of protein isolates

The solubility of protein isolates as a function of pH was determined as described by Ambigaipalan and Shahidi (2015) with slight modifications. Protein isolates were dispersed in distilled water to obtain a final concentration of 10 mg protein/ml of solution. Each dispersion adjusted to pH 2, 5, 8, and 12 by adding 1M HCl and 1M NaOH. Mixtures were stirred for 30 min and centrifuged at 7,500g for 15 min. The protein contents in the supernatants were analysed by the biuret method. Total protein content was determined after solubilization of the sample in 0.5 N NaOH. Protein solubility (%) was calculated according to Eq. 1.

$$\text{Protein Solubility}(\%) = \frac{\text{Protein content in Supernatant}}{\text{Total Protein content in sample}} \times 100 \qquad \text{(Eq.1)}$$

3.2.2.4 Oil holding capacity (OHC) and water (WHC) holding capacity

The OHC was estimated by the centrifuge method reported by Wasswa et al. (2007) with some modifications. Protein isolates (0.1 g) were placed at previously weighted

centrifuge tubes and dissolved in 10 mL of water and 10 mL of oil, respectively. Mixtures were vortexed for 30 sec and allowed to stand for 1h at room temperature before being centrifuged at 5000g for 30 min. The supernatant volumes were measured. The WHC and OHC were determined using Eq. 2.

$$\text{WHC/OHC} = \frac{(\text{Original volume} - \text{Supernatant volume})}{\text{Total protein content in sample}} \times 100 \qquad \text{(Eq. 2)}$$

3.2.2.5 Emulsifying properties of protein isolates

The emulsifying activity (EAI) and stability (ESI) of the protein isolates were estimated according to Chen et al. (2019) with minor modifications. Protein solutions (0.2%) were adjusted to pH 2, 4, 6, 8, and 10 with 1M HCl and 1M NaOH. Soybean oil (10 mL) was added to each protein solution (30 mL). The mixture was homogenized at 13,500 rpm for 2 min by using a homogenizer (Polytron Kinematica PT- 3000). Then, 50 μL of the emulsion were pipetted at 0 and 10 min and subsequently diluted 100-fold using 0.1% SDS solution. The absorbance of the mixture was measured at 500 nm by diode array spectrophotometer (Agilent, Palo Alto, CA, USA). EAI and ESI were calculated by Eq. 3 and 4.

$$\text{EAI}\left(\frac{\text{m2}}{\text{g}}\right) = \frac{(2 \times 2.303 \times A_0 \times N)}{(c \times \phi \times 104 \times L)} \qquad \text{(Eq. 3)}$$

$$\text{ESI(min)} = \frac{(A_0 \times \Delta t)}{(A_0 - A_{10})} \qquad \text{(Eq. 4)}$$

where A_0 = absorbance measured at 0 min, A_{10} = absorbance measured at 10 min, N = dilution factor, c = protein concentration in aqueous phase (g/mL) and ϕ = oil volume fraction.

3.2.2.6 Foaming properties of protein isolates

The foaming ability and foam stability of protein isolates were determined according to Shahidi et al. (1995), with slight modifications. Protein solutions (0.5%) were adjusted to pH 2, 4, 6, 8, and 10 using 1M HCl and 1M NaOH. Twenty milliliters of each solution were homogenized in a 50 mL cylinder at a speed of 16,000 rpm for 2 min using a homogenizer (Polytron PT3000, Kinematica, Littau, Switzerland). The total volume of the whipped mixture was measured at 0 and 30 min. The foaming ability was expressed as foam expansion at 0 min, while foam stability was expressed as foam expansion after 30 min of whipping. Foam expansion and foam stability were calculated according to Eq. 5 and 6.

$$\text{Foam expansion}(\%) = \left[\frac{(A-B)}{B}\right] \times 100 \qquad \text{(Eq. 5)}$$

$$\text{Foam stability}(\%) = \left[\frac{(A_{30}-B)}{(A_0-B)}\right] \times 100 \qquad \text{(Eq. 6)}$$

where A_0 = volume after whipping (ml); A_{30} = volume after 30 min of whipping (ml) and B = volume before whipping (ml)

3.2.2.7 Surface hydrophobicity

The surface hydrophobicity (H_0) of the protein isolates was determined as described by Chelh, Gatellier and Santé-Lhoutellier (2006). Bromophenol blue (BPB) solution (1 mg/mL in distilled water) was added to 1 mL of protein isolate samples (5 mg/mL) prepared in 20 mM phosphate buffer (pH 7.0) and vigorously shaken. A control was prepared using 200 μL BPB solution devoid of sample and 1 mL of phosphate buffer (20 mM, pH 7.0). Samples and control were kept under agitation at room temperature for 10 min followed by centrifugation (2000 g, 15 min). The absorbance of the supernatant (1:10 diluted) was recorded at 595 nm using a diode array spectrophotometer (Agilent). Phosphate buffer (20 mM, pH 7.0) was used as the blank. The hydrophobicity of the protein samples was calculated from the BPB bound method according to Eq. 7.

$$\text{BPB bound}(\mu g) = 200\mu g \times \frac{(A_c - A_s)}{A_c} \qquad \text{(Eq. 7)}$$

where A_c is the absorbance of the control and A_s is the absorbance of the sample.

3.2.2.8 Free sulfhydryl (SH) and disulfide bonds measurements

Determination of free SH (SH_F) content was measured according to the Ellman's reagent method established by Patrick and Swaisgood (1976), with some modifications. Briefly, 60 mg of protein solution were mixed with 10 mL of Tris-Gly buffer (8 M urea, 0.5% (w/v) sodium dodecyl sulfate, 0.086 M Tris, 0.09M glycine and 0.004M EDTA, pH 8.0). Then 80 μL of Ellman's reagent were added to 2 mL of sample solution. After mixing for 5 min, the absorbance of the mixture was read at 412 nm using a diode array

spectrophotometer (Agilent). The disulfide bonds (SS) were measured according to the method by Synowiecki and Shahidi, (1991), with some modifications. Protein solution (2 mL) was mixed with 2.4 μL of β- mercaptoethanol for 2 h to determine the total SH group (SH_T) and protein was precipitated using trichloroacetic acid (TCA, 12%, w/v) for 1 h. Then, the mixture was centrifuged at 10,000g for 10 min, with the precipitation step being repeated three times. Recovered precipitate was resolubilized in 2 mL of Tris-Gly buffer. Ellman's reagent (80 μL) was added to 2 mL of protein solution and the absorbance was measured at 412 nm. SS was determined using SH_F and SH_T according to Eq. 8 and 9.

$$SH\left(\frac{\mu mol}{g}\right) = 73.53 \times A\ 412 \times \left(\frac{D}{C}\right) \qquad (Eq.\ 8)$$

$$SS\left(\frac{\mu mol}{g}\right) = \frac{(SHT\text{-}SHF)}{2} \qquad (Eq.\ 9)$$

where C is the protein concentration (mg/mL) and D is the dilution factor.

3.2.2.9 Determination of functional groups of protein isolates

Total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy was performed using a Fourier transform infrared spectrometer (Tensor 27, Bruker, Germany) according to the method described by Tran, Miranda, Mouradov and Adhikari (2020), with minor modifications. The FT-IR spectra of the samples was recorded within the wave range of 650- 4000 cm^{-1} with a resolution of 2 cm^{-1}, and 32 scans were made for the generation of each spectrum.

3.2.3 Production of protein hydrolysates

Freeze-dried protein isolates were mixed with deionized water (1:4, w/v) and enzymes were added according to the preselected ratio of enzyme to protein for each treatment (0.3 AU/g for Alcalase; 50 LAPU/g for Flavourzyme and 840 UHb /g for Corolase 7089). The samples were hydrolysed batchwise with Alcalase (A, pH 8, 50 °C) for 4h, Flavourzyme (F, pH 7, 50°C) for 4 h, and Corolase 7089 (C, pH 8, 52 °C) for 4 h. Enzyme combination treatments were conducted by hydrolysing first with Corolase for 2h (pH 8, 52 °C) followed by adding Flavourzyme (pH 7, 50°C) for an additional 2h. Similar sequential addition were conducted for Alcalase and Flavourzyme, where samples were hydrolysed with Alcalase for 2h (pH 8, 50 °C), followed by the addition of Flavourzyme for 2h (pH 7, 50°C). Conditions were constantly monitored and maintained throughout the process by the addition of 4M NaOH. Upon completion of the incubation periods the reactions were terminated by heating the mixture at 90°C for 10 min to inactivate the enzyme.

The degree of hydrolysis (DH, %) was determined by the TNBS (trinitrobenzenesulfonic acid acid) method (Alder-Nissen, 1979). The hydrolysates were centrifuged at 10,000 g for 15 min and the recovered supernatants were freeze-dried and stored at -20 °C until further analysis

3.2.4 Bioactive properties of protein hydrolysates

Figure 3.3 summarizes the experimental design for the antioxidant activity tests conducted for sea cucumber protein hydrolysates.

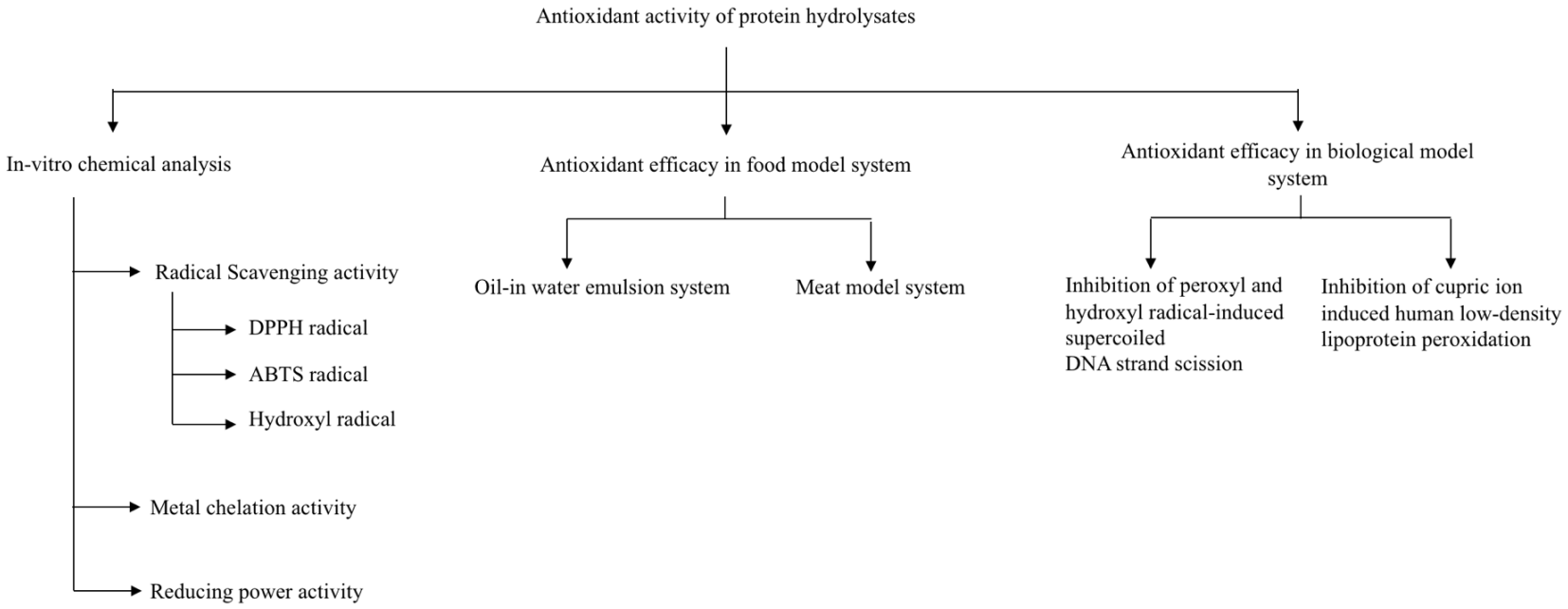


Figure 3.3 Experimental design for antioxidant analysis of sea cucumber protein hydrolysates

3.2.4.1 DPPH radical scavenging activity for protein hydrolysates

The DPPH (2,2-diphenyl-1,1-picrylhydrazyl) radical scavenging activity was determined using a Bruker-E-scan electron paramagnetic resonance (EPR) spectrometer (Bruker E-scan, Bruker Biospin Co., Billericia, MA, USA) according to the method described by Chandrasekara and Shahidi (2011), with minor modifications. Protein hydrolysates (250 μL 0.5 mg/mL) were added to 1 mL of 0.3 mM DDPH methanolic solution. The mixture was immediately vortexed and incubated in the dark for 30 min at room temperature. Then, the mixtures (1 mL) were introduced into the sample cavity of the EPR spectrometer and the spectrum was recorded after 10 min. Trolox was used to prepare the standard curve (50–500 μM in methanol). The operating parameters of the EPR spectrometer were set as follows: 1.86 G modulation amplitude, 5.02 $\times 10^2$ receiver gain, 2.621s sweep time, 8 scans, 100.00 G sweep width, 3495.53 G center field, 5.12 ms time constant, 9.795 GHz microwave frequency, 1.86 G modulation amplitude, and 86.00 kHz modulation frequency. DPPH radical scavenging capacity, expressed as micromoles (μM) of Trolox equivalents (TE) per milligram of protein hydrolysate, was calculated according to Eq. 10.

$$\text{DPPH radical scavenging capacity}(\%) = \frac{(\text{EPR signal intensity for the control} - \text{EPR signal for the sample})}{\text{EPR signal intensity for the control}} \times 100 \quad \text{(Eq. 10)}$$

3.2.4.2 ABTS radical scavenging activity for protein hydrolysates

The 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) scavenging activity of protein hydrolysates was determined as described by John and Shahidi (2010). The protein hydrolysates and reagents were prepared in 100 mM phosphate buffer (PBS) containing 0.15 M NaCl (pH 7.4). ABTS solution was prepared by mixing 2.5 mM 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) with 2.5 mM ABTS in a 1:1(v/v) ratio. The mixture was heated for 20 min at 60 °C and stored in the dark at room temperature. Protein hydrolysates (40 μL,1 mg/mL) were mixed with the ABTS solution (1.96 mL) and the mixture was allowed to react for 6 min prior to reading the absorbance at 734 nm. A blank was prepared in the same manner using distilled water instead of sample. A standard curve was constructed using different concentrations of Trolox (0-1000 μM). ABTS radical scavenging activity, expressed as micromoles (μM) of Trolox equivalents (TE) per milligram of protein hydrolysates was calculated according to Eq. 11.

$$\text{ABTS radical scavenging ability}(\%) = \frac{(\text{Absorbance of the blank} - \text{Absorbance of the sample after 6 min})}{\text{Absorbance of the blank}} \times 100 \quad \text{(Eq. 11)}$$

3.2.4.3 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging capacity was determined using an EPR spectrometric (Bruker E-scan, Bruker Biospin Co., Billericia, MA, USA) method as described by Chandrasekara and Shahidi (2011), with slight modifications. The samples

were dissolved in deionized water to obtain a final concentration of 10 mg/mL. The sample (200 μL) was mixed with 10 mM H_2O_2 (200 μL), 17.6 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO; 400 μL) and 10 mM $FeSO_4$ (200 μL). The mixture was allowed to react for 3 min at room temperature, then injected into the sample cavity of the EPR spectrometer. Deionized water was used as the control. The EPR spectra was recorded and Trolox (0-50 μM) was used to prepare the standard curve. The hydroxyl radical scavenging capacity, expressed as micromoles (μM) of Trolox equivalents (TE) per milligram of protein hydrolysate, was calculated using Eq. 12.

$$\text{Hydroxyl radical scavenging capacity}(\%) = \frac{(\text{EPR signal intensity for the control-EPR signal intensity for the sample})}{\text{EPR signal intensity for the control}} \times 100 \quad \text{(Eq. 12)}$$

3.2.4.4 Reducing power

The reducing power of sea cucumber protein hydrolysates was evaluated according to the method described by Cumby et al. (2008), with minor modifications. Phosphate buffer (0.2M, pH 6.6) was used to dissolve sea cucumber protein hydrolysates (0.5 mg/mL). One milliliter of sea cucumber protein hydrolysate was mixed with 2.5 mL of 1% potassium ferricyanide solution and the mixture was incubated at 50 °C for 20 min. Then, 2.5 mL of 10% trichloroacetic acid (TCA) was added and centrifuged for 10 min at 1,000 g. After centrifugation, 2.5 mL of supernatant was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride solution. The reaction was allowed to react for 10 min and the absorbance of the solution was measured at 700 nm. The control was prepared without the

addition of hydrolysates while the blank was contained only protein hydrolysates. A standard curve was built using varying concentrations (0-1,000 μM) of Trolox and the reducing power was expressed as micromoles (μM) of Trolox equivalents (TE) per milligram of protein.

3.2.4.5 Metal chelation activity

Ferrous ion chelating activity was determined according to the method of Chandrasekara and Shahidi (2010), with minor modifications. Two hundred microliters of sea cucumber protein hydrolysates (0.5 mg/mL) was mixed with 1.74 mL of distilled water, 20 μL of $FeCl_2$ (2 mM) and 40 μL of ferrozine (5 mM) and incubated at room temperature for 10 min. The absorbance was recorded at 562 nm. The control was prepared using distilled water and a standard curve was built using trisodium salt of ethylenediaminetetraacetic acid (Na_3EDTA). Metal ion chelating ability (%) was calculated using Eq.13.

$$\text{Metal ion chelation activity}(\%)=\left\{1-\left(\frac{\text{Absorbance of the sample}}{\text{Absorbance of the control}}\right)\right\}\times 100 \qquad \text{(Eq. 13)}$$

3.2.4.6 Antioxidant activity in cooked comminuted meat model system

The cooked comminuted meat model system was prepared according to the method of Wettasinghe and Shahidi (1999). Fresh ground pork (40g) was mixed with 10 mL of distilled water in a Mason jar and protein hydrolysates (200 ppm) were added to the

mixture. Butylated hydroxytoluene (BHT) was used as a positive control and a negative control (no protein hydrolysates) was also prepared. All samples were cooked in a thermostated water bath (80 °C) for 40 min with gentle stirring every 5 min. The samples were cooled to room temperature prior to being transferred into plastic bags for storage at 4 °C. Samples were randomly taken out according to designated time interval (0, 3, 5, 7, 10, and 14 days) for analysis of thiobarbituric acid reactive substances (TBARS) described by Wijeratne, Abou-Zaid, and Shahidi (2006). The TBARS values were calculated using a standard curve of malondialdehyde (MDA), using MDA precursor, and expressed as mg MDA equivalents per kilogram of sample.

3.2.4.7 Antioxidant activity in oil-in-water emulsions

The antioxidant activity of protein hydrolysates in oil-in-water emulsion was evaluated using a β-carotene-linoleate model system according to the method described by Ambigaipalan and Shahidi (2015). Briefly, 10 mg of β-carotene were dissolved in 10 mL of chloroform. Then, 1.2 mL of the β-carotene solution were pipetted into a 50 mL round-bottom flask which contained linoleic acid (40 mg) and Tween 40 (400 mg). Chloroform was subsequently removed under a stream of nitrogen before the addition of oxygenated distilled water (100 mL) to the flask. The mixture was then vigorously stirred for 30 min and an aliquot of 4.5 mL was mixed with 0.5 mL of protein hydrolysate (0.5 mg/mL in distilled water). For each sample, a blank was prepared without β-carotene and control without protein hydrolysates was also prepared. Trolox (100 ppm) was used as a positive control. Immediately after the addition of the emulsion, the absorbance was read at 470 nm.

The tubes were then placed in a shaking water bath (50 °C) and the absorbance was continuously read over a 105 min period at 15 min intervals. Antioxidant activity of protein hydrolysates in an oil-in-water emulsion system was calculated according to Eq. 14.

$$\text{Antioxidant activity}(\%)=\left[\frac{(A_0-A_t)}{(A0_0-A0_t)}\right]\times 100 \qquad \text{(Eq. 14)}$$

where A_0 and At are corrected absorbance values for protein hydrolysates at time 0 and after incubation, and $A0_0$ and $A0_t$ are absorbance value for control at time t and after incubation respectively.

3.2.4.8 Inhibition of peroxyl and hydroxyl radical-induced supercoiled DNA strand scission

The inhibitory activity of protein hydrolysates against DNA strand scission caused by the action of hydroxyl and peroxyl radicals was determined according to Chandrasekara and Shahidi (2011). Supercoiled plasmid DNA (PBR 322) was dissolved in 0.5 M PBS (pH 7.4) to a final concentration of 50 µg/mL while protein hydrolysates and carnosine standard were dissolved in distilled water. For the determination of peroxyl radical-induced DNA oxidation, 2 µL of protein hydrolysates (6 mg/mL) were mixed with 4 µL AAPH [2, 2′-azobis (2-aminopropane) dihydrochloride, 7 mM], 2 µL of PBS (0.1 M) and, and 2 µL of DNA (50 µg/mL). To produce hydroxyl radical 2 µL of $FeSO_4$ (0.5 mM), and 2 µL of H_2O_2 (0.5 mM) were added to the mixture of protein hydrolysates (2 µL, 0.1 mg/mL), PBS (2 µL, 0.1 M) and DNA (2 µL, 50 µg/mL). The mixture was incubated at 37 °C for 1h in the dark prior to the addition of 2 µL of loading dye consisting of 0.25% bromophenol blue, 0.25% xylene cyanol and 50% glycerol in distilled water. A control (DNA only), and a

blank (DNA + free radicals devoid of protein hydrolysates) were prepared for each set of tested samples. Ten microliters of the mixtures were then loaded into agarose gel (0.7% w/v), prepared in Tris−acetic acid−EDTA (TAE) buffer (40 mM Tri-acetate containing 1 mM EDTA, pH 8.5), and stained with SYBR safe. Gel electrophoresis was conducted in a horizontal submarine gel electrophoresis apparatus (Owl Separation Systems Inc., Portsmonth, NH, USA) at 80 V for 90 min. Alpha-Imager gel documentation system (Cell Biosciences, Santa Clara, CA, USA) was used to visualize the bands under trans-illumination of UV light. The protective effect of protein hydrolysates was determined using the retention percentage of supercoiled DNA strand according to Eq. 15.

$$\text{DNA retention}(\%) = \frac{\text{Area of supercoiled DNA with oxidative radical and protein hydrolysate}}{\text{Area of supercoiled DNA in control}} \times 100 \quad \text{(Eq. 15)}$$

3.2.4.9 Inhibition of cupric ion- induced human low-density lipoprotein (LDL) peroxidation

Protein hydrolysates were evaluated for their inhibitory effect of cupric ion-induced human LDL peroxidation according to the method described by Liyana-Pathirana and Shahidi (2006), with slight modifications. Human LDL cholesterol (5 mg/mL) was dialysed in 10 mM phosphate buffer (PBS; pH 7.4, 0.15 M NaCl) at 4 °C for 18 h. The dialysed and diluted LDL (0.04 mg/mL) was mixed with protein hydrolysate solutions (0.1 mg/mL) and pre-incubated at 37 °C for 15 min. The oxidation reaction was then initiated by adding 100

μM cupric sulfate, followed by incubation at 37 °C for 12 h. The resultant conjugated dienes from human LDL oxidation were measured at 234 nm using a spectrophotometer (Agilent) between 3 h intervals until the end of the incubation period. For each sample, an appropriate blank was prepared devoid of LDL or CuSO4 and carnosine was used as a positive control. The inhibitory effect of the protein hydrolysates on the formation of conjugated dienes was calculated using Eq. 16.

$$\% \text{ inhibition CD} = \left[\frac{(\text{Abs control-Abs sample})}{(\text{Abs control-Abs native LDL})}\right] \times 100 \qquad \text{(Eq. 16)}$$

where Abs control is the absorbance of LDL with $CuSO_4$ and PBS; Abs sample is the absorbance of LDL with $CuSO_4$ and sample or standard, and Abs native LDL is absorbance of LDL and PBS only.

3.2.4.10 Angiotensin I converting enzyme (ACE) Inhibitory activity

Sea cucumber protein hydrolysates exhibiting the highest antioxidant activities from each organ were used to determine the ACE inhibitory activity. The inhibitory activity was determined according to the method of Cushman and Cheung (1971), with slight modifications. Sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES)-HCl buffer (50 mM) containing 300 mM NaCl (pH 8.3) was used to dissolve samples and ACE. Twenty-five microliters of ACE solution (0.25 unit/mL) was added to each of the sea cucumber protein hydrolysates (50 μL, 5 mg/mL), and the mixture was pre-incubated at 37 °C for 5 min. Then, the reaction was initiated by adding 50 μL of hippuryl-L-histidyl-L-

leucine (HHL, 6 mg/ mL) solution to the mixture and incubated at 37 °C for 15 min. To terminate the enzymatic reaction, HCl (125 µL, 1M) was added and formed hippuric acid, which was extracted with ethyl acetate (1 mL). The mixture was vortexed for 1 min prior to the centrifugation at 1,200 × g for 5 min using an Eppendorf centrifuge (model 5415, Hamburg, Germany). Collected supernatant (1mL) was placed in boiling water to remove ethyl acetate. The remaining hippuric acid in the tube as dissolved in distilled water (1 mL) and the absorbance was recorded at 228 nm. The control was prepared by using HEPES-HCl buffer (50 mM) containing 300 mM NaCl (pH 8.3) devoid of sample. The sample blank and control blank were run in the same manner. For the control blank, ACE solution was added into the reaction before addition of 1 M HCl. The ACE- inhibitory activity (%) was determined according to Eq. 17.

ACE inhibitory activity(%)=

$$\left\{1-\frac{(\text{Absorbance of sample-Absorbance of sample blank})}{(\text{Absorbance of control-Absorbance of control blank})}\right\}\times 100 \qquad \text{(Eq. 17)}$$

3.2.5 Physio-chemical properties of protein hydrolysates

3.2.5.1 Color measurement of freeze-dried hydrolysates

The color of the freeze-dried hydrolysate was measured by Hunter lab color meter (Hunter Associates Laboratory Inc, Reston, VA, USA) and reported using CIE system, where L, a and b parameters indicate lightness, redness and yellowness, respectively.

3.2.5.2 Solubility of protein hydrolysates

The solubility of protein hydrolysates was determined at different pH values, following the method described by Ambigaipalan and Shahidi (2015) with slight modifications. Protein hydrolysates were dispersed in distilled water to obtain the final concentration of 10 mg protein/ml. The pH of each dispersion was adjusted to 2, 5, 8, and 12 by adding 1M HCl and 1M NaOH. Mixtures were stirred for 30 mins and centrifuged at 7,500g for 15 min. The protein content in the supernatants was analysed using the biuret method. Total protein content was determined after solubilization of the sample in 0.5 M NaOH. Protein solubility was calculated according to Eq. 1 as mentioned in the section 3.2.2.3.

3.2.5.3 Determination of water holding capacity of protein hydrolysates using meat model system

The water holding capacity of sea cucumber protein hydrolysates was evaluated according to the method reported by Shahidi and Synowiecki (1995). Ground pork (8.5 g) and distilled water (1.5g) was mixed in a pre-weighed 50 mL centrifuge tube to prepare the meat model system. Sea cucumber protein hydrolysates (0.5%, w/w) were added to the system and mixed thoroughly. A control was prepared devoid of any sample whereas sodium tripolyphosphate (0.3%, w/w) was used as positive control. The mixture was placed in a cold room for 1 h before cooking at 95°C in a water bath for 1 h. The cooked homogenates were cooled under a stream of cold tap water and, subsequently, the drip volume was measured using a filter paper. The final weight of the homogenate was recorded, and the drip volume was determined using the weight loss of the meat model after

cooking. The water holding capacity of protein hydrolysates was reported as decrease of drip volume against the control.

3.2.6 Identification of bioactive peptides using proteomics and bioinformatics

Samples with the highest antioxidant activities were used for further analysis of bioactive peptides. The amino acid composition of the samples was analysed according to the method described in the section 3.2.2.1.

3.2.6.1 Molecular weight analysis by liquid chromatography- mass spectrometry (LC-MS/MS)

LC-MS/MS analysis was carried out at the Analytics, Robotics and Chemical Biology Centre (SPARC BioCentre), The Hospital for Sick Children, Toronto, ON, Canada using Q - Exactive Orbitrap analyzer outfitted with a nanospray source and EASY-nLC nano-LC system (Thermo Fisher, San Jose, CA, USA), as reported by Udenigwe, Udechukwu, Yiridoe, Gibson and Gong (2016). The binary mobile phase consisted of aqueous formic acid (0.1%, v/v; eluent A) and formic acid in HPLC grade acetonitrile (0.1%, v/v; eluent B). Samples were dissolved in 0.1 % formic acid loaded to an Acclaim PepMap 100 C18 (75 µm x 2 cm, particle diameter 2 µm) pre-column (Thermo Fisher). The samples were separated on an analytical column PepMax RSLC EASY-Spray C18 (75 µm x 50 cm) (Thermo Fisher) at a pressure of 900 bar with a flow rate of 250 nl/min using gradient set up as 3- 35% of eluent A (0.1% formic acid) over 50 min, and then keeping 100% eluent B over 10 min. Eluted peptides were introduced by nano-electrospray into the

Q-Exactive mass spectrometer (Thermo Fisher). MS scans were acquired by the Orbitrap mass analyser with a resolution of 60,000 for 1 mass spectrometry (scan range from 400 to 1600 m/z) with automatic gain control (AGC) target of 3e6 and maximum ion injection time of 150 ms. The instrument method then followed by performing 10 data dependent tandem MS (MS/MS) scans with a resolution of 7,500 an AGC of 1e6, maximum injection time of 22 ms and one microscan. The intensity threshold to trigger a MS/MS scan was set to 4 with 30% normalised collision energy. The dynamic exclusion was applied using a setting of 8s. PEAKS X+ software (Bioinformatic Solutions, Waterloo, ON, Canada) was used to perform the data analysis.

3.2.6.2 *In silico* prediction of bioactive potential of identified peptides from protein hydrolysates samples

Peptides identified using PEAKS X+ software were further analysed for their bioactive potential using *in silico* tools (Figure 3.4).

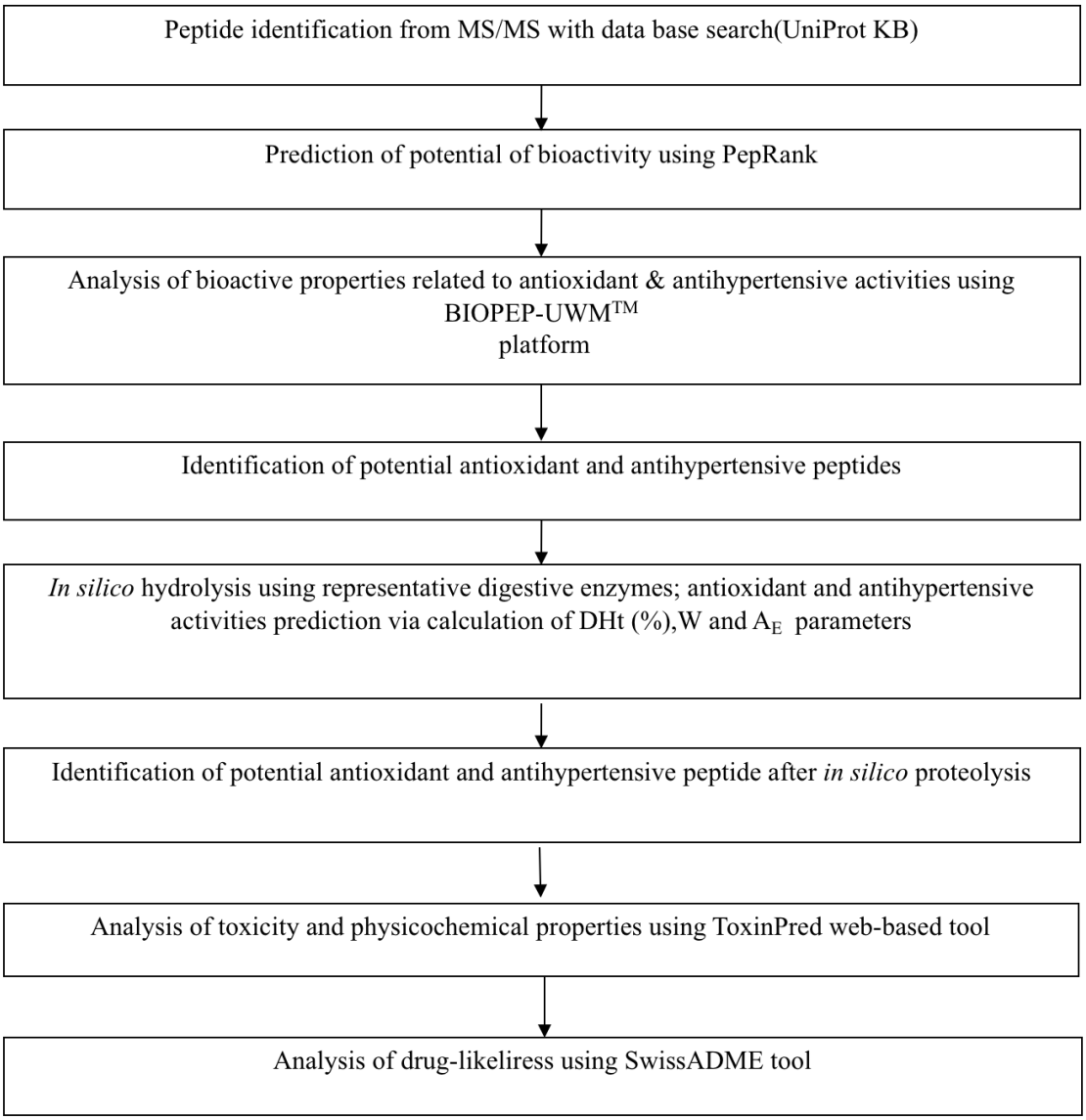


Figure 3.4 Virtual screening process of bioactive peptides with bioinformatics tools

Peptides were screened according to their bioactive potential using PepRank (http://bioware.ucd.ie/~compass/biowareweb/), which predicts the probability (between 0 and 1) of the peptide being bioactive, as described by Mooney, Haslam, Pollastri, and Shields (2012). The threshold of 0.9 was selected to reduce the number of false positives.

Selected peptides were analysed for their bioactive properties related to antioxidant and antihypertensive activities using BIOPEP-UWM^{TM} data base (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) of bioactive peptides. The identified sequences with ACE inhibitory peptides were subjected to *in silico* hydrolysis using the enzyme(s) action feature of BIOPEP-UWM^{TM} using pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) as representative digestive enzymes as described by Ji, Xu, Udenigwe, and Agyei (2020). The quantitative parameters during the proteolysis simulation were calculated including the theoretical degree of hydrolysis (DH_t), the frequency of the release of fragments within the given activity by selected digestive enzymes (A_E), and the relative frequency of the release of fragments with a given activity by selected enzymes (W) using BIOPEP-UWM^{TM} platform. The *in silico* parameters were calculated by Eq 18, 19, and 20.

$$DH_t = \left(\frac{d}{D}\right) \times 100\% \qquad \text{(Eq. 18)}$$

where:

d – number of hydrolyzed peptide bonds and D- total number of peptide bonds in a protein chain

$$A_E = \frac{d}{N} \qquad \text{(Eq. 19)}$$

where:

d- number of fragments with specific bioactivity in a protein sequence that can be released by enzymes and N-number of amino acid residues of protein

$$W = \frac{A_E}{A} \qquad \text{(Eq. 20)}$$

where: A_E -frequency or release of fragments with given activity by selected enzymes and A- frequency of occurrence of bioactive fragments in a protein sequence

3.2.6.3 Toxicity and physicochemical properties of bioactive peptides released after *in silico* proteolysis

The potential toxicity, hydrophobicity, hydrophilicity, charge, isoelectric point and molecular weight of the peptides released after simulated digestive proteolysis were predicted using ToxinPred (https:// webs.iiitd.edu.in/raghava/toxinpred/index.html) web-based application according to the method described by Gupta et al. (2013).

3.2.6.4. *In silico* analysis of absorption, distribution, metabolism and excretion (ADME) profile of bioactive peptides

In silico analysis of drug-likeliness for identified sea cucumber derived-peptides was evaluated based on absorption, distribution, metabolism and excretion parameters using SwissADME tool (http://www.swissadme.ch/index.php#) as explained by Daina, Michielin and Zoete (2017).

3.2.7 Statistical analysis

All the experiments were carried out in triplicates and data were reported as mean ± standard deviation. One-way ANOVA was performed, and means were compared by Tukey's HSD test ($P < 0.05$) using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

CHAPTER 4

PHYSICOCHEMICAL PROPERTIES OF SEA CUCUMBER PROTEIN ISOLATES

4.1 Introduction

Global demand for seafood proteins products has been increasing rapidly due to awareness about their health effects (Shahidi and Ambigaipalan, 2015). However, seafood production is not sufficient to meet the total requirement of marine proteins. As a result, there has been a growing interest in research to identify the potential of maximizing the yield of proteins derived from seafoods including the processing by-products (Abdollahi and Undeland, 2019). In this regard, the role of aquaculture in meeting the increasing demand must also be taken into consideration. Improving desired functional properties of proteins from seafood and its processing discards has been the focus of research in recent years. In this regard, the pH-shift processing method that includes acid- alkaline solubilization and isoelectric precipitation has been recognized as an efficient way for recovering proteins from underutilized marine species and fish processing by-products (Kristinsson and Liang, 2006). Unique physicochemical and functional properties of recovered protein isolates promote their application in both food and nutraceutical industries. Proteins isolated from aquatic sources possess better nutritional properties and distinct flavor quality than proteins derived from plants and terrestrial animals (Shahidi, 1994; Han et al., 2019). This chapter focuses on the compositional analysis and physicochemical properties of sea cucumber protein isolates recovered from body wall,

flower and internal organs to evaluate their potential applicability as functional food ingredients.

4.2 Yield and amino acid composition

The yield of protein isolates obtained for body wall and flower were in the range of 70-80% but somewhat lower for internal organs (65-70%). This demonstrates the potential commercial viability of the process.

In general, the amino acid profile of protein isolates reflects their nutritional value (Wu et al., 2016). Table 4.1 represents the amino acid profiles of sea cucumber protein isolates along with the whole sea cucumber powder. The major amino acids of the protein isolates prepared from sea cucumber were glutamic acid, aspartic acid, and arginine, which are non-essential amino acids (NEAA). Similar findings were reported in previous studies on Atlantic sea cucumber by Zhong et al. (2007) and Mamelona et al. (2010a), indicating the abundance of glutamic acid in body wall and high content of glutamic acid and aspartic acid in viscera. A high level of glutamic acid has the potential to stimulate immune and central nervous systems whereas proteins rich in aspartic acid could be involved in hormonal regulatory activities related to the nervous system (Deng et al., 2019).

Leucine was the predominant essential amino acid (EAA) present in the three samples of body wall, flower and internal organs. The total EAA content of the protein isolate in these samples, 42.72, 43.32, and 45.52 mole%, respectively, were higher than that of whole sea cucumber powder prior to isolation of proteins (34.00 mole%). These results imply that sea cucumber protein isolates recovered using pH-shifting process can be used as enriched sources of EAA. Generally, protein quality is determined by the amount and quantity of EAA. Thus, high content of EAA indicates the high quality of proteins (Surasani, 2018). The EAA content observed in sea cucumber protein isolates was higher

than some of the reported aquatic protein isolates, including scallop female gonads (Wu et al., 2016) and sea urchin gonads (Shang et al., 2020), where the EAA contents were 41.27 and 38.2 %, respectively.

The ratio of EAA/NEAA is considered as an indicator for protein quality of different dietary protein sources (Shahidi, 1994). The ratio of EAA/NEAA in body wall (0.75), flower (0.76) and internal organs (0.84) were comparatively higher than that of whole sea cucumber (0.52). Based on the World Health Organization /Food and Agriculture Organization (WHO/FAO, 2007) recommendations, the ideal ratio of EAA/NEAA is above 0.6. Thus, protein isolates from body wall, flower and internal organs were of better nutritional quality. The observed EAA/NEAA ratio in our study was higher than those reported for immature pollock roe isolates (0.5) (Bechtel, Chantarachoti, Oliveira, and Sathivel, 2007), sea cucumber (*Stichopus japonicus*) gut protein isolates (0.65) (Du et al., 2019), sea urchin gonad isolates (0.62) (Shang et al., 2020) and lower than those of ponyfish (0.95) (Özyurt, Şimşek, Karakaya, Aksun, and Yeşilsu, 2015) and yellow fin tuna (0.98) (Lee et al., 2016).

In addition, sea cucumber protein isolates had a higher content (17.79%, 18.28%, 18.83% from body wall, flower and internal organs) of total branched-chain amino acids (BCAA) compared to the whole sea cucumber (13.45%). BCAAs including leucine, isoleucine, and valine have shown regulatory impact on blood sugar level and reduced physical and mental fatigue (Zhang et al., 2020). Moreover, total content of aromatic amino acids (AAA) in protein isolate samples were also greater than that of whole sea cucumber. It has been shown that greater BCAA/AAA ratios have a positive influence on muscle metabolism and protein hemostasis (Du et al., 2020). As shown in Table 4.2, amino acid

profiles of sea cucumber protein isolates fulfill the recommendations of the WHO/FAO (2007) of or most essential amino acids in food products. Therefore, overall amino acid compositional analysis of sea cucumber protein isolates revealed that they can be considered as rich sources of EAA that could significantly contribute to better nutrition and health promotion.

Table 4.1 Amino acid composition of protein isolates from different body parts of the North Atlantic sea cucumber and whole sea cucumber powder (Mole %)

Amino acid with single letter code	Whole	Body wall	Flower	Internal organs
Essential AA				
Histidine (His)- H	1.68	2.28	2.25	2.23
Isoleucine (Ile)- I	3.48	4.77	4.87	5.01
Leucine (Leu)- L	5.65	7.40	7.69	7.85
Lysine (Lys)- K	6.12	6.96	7.04	7.78
Methionine (Met)- M	1.95	2.69	2.61	3.05
Phenylalanine (Phe)- F	4.21	4.93	5.14	5.47
Threonine (Thr)-T	5.36	6.36	6.24	6.60
Tryptophan (Trp)- W	1.22	1.71	1.75	1.57
Valine (Val) - V	4.33	5.63	5.72	5.96
Total EAA	34.00	42.72	43.32	45.52
Non EAA				
Alanine (Ala)- A	5.39	4.41	4.25	4.13
Arginine (Arg)- R	9.89	7.76	7.38	7.59
Aspartic acid +Asparagine (Asp+Asn)- D+N	9.56	8.17	9.97	8.88
Cystine (Cys)- C	1.32	2.27	2.20	1.70
Glutamic acid +Glutamine (Glu+Gln)- E+Q	15.41	13.23	13.26	12.64
Glycine (Gly)- G	9.28	5.34	4.63	4.42
Proline (Pro)- P	5.51	5.08	4.57	4.23
Serine (Ser)- S	5.86	6.00	5.50	5.73
Tyrosine (Tyr)-Y	3.76	5.03	4.91	5.16
Total NEAA	66.00	57.28	56.68	54.48
EAA/ NEAA	0.52	0.75	0.76	0.84
Total polar AA	58.98	58.05	58.77	58.31
Total hydrophobic AA	41.02	41.95	41.23	41.69
Total Branched chain AA	13.45	17.79	18.28	18.83
Total Aromatic AA	16.31	19.65	18.86	19.19

Table 4.2 Comparison of essential amino acid composition of protein isolates from different body parts of the North Atlantic sea cucumber and World Health Organization /Food and Agriculture Organization (WHO/FAO, 2007) recommendations (g/100g)

Amino Acid	WHO/FAO	Body wall	Flower	Internal organs
Histidine (His)	1.60	1.52	1.37	1.01
Threonine (Thr)	0.90	4.24	3.80	2.99
Valine (Val)	1.30	3.75	3.48	2.71
Methionine (Met)	1.70	1.79	1.59	1.38
Isoleucine (Ile)	1.30	3.18	2.97	2.27
Leucine (Leu)	1.90	4.93	4.69	3.56
Phenylalanine+ Tyrosine (Phe+Tyr)	1.90	10.39	9.61	7.53
Lysine (Lys)	1.60	4.64	4.29	3.53
Tryptophan (Trp)	0.50	1.14	1.07	0.71

4.3 Color of sea cucumber protein isolates

Color is considered as a crucial quality attribute for determining the consumer acceptance of food (Thiansilakul et al., 2007; Gehring et al., 2011). Color (L, a, b) measurements for sea cucumber protein isolates are presented in Table 4.3. Compared to soy protein isolate, all sea cucumber samples showed significantly lower whiteness (L) and a higher redness (a) and yellowness (b). Among the sea cucumber samples, whiteness of body wall protein isolates (57.80±0.36) was significantly higher than those of flower (43.01±0.40) and internal organ isolates (41.91±0.98). As L values correspond to the sample with lighter color, body wall isolates were found to be substantially lighter than flower and internal organs of sea cucumber. The higher L values or the whiteness of the protein isolates may be associated with the retention of connective tissues (Kristinsson, Theodore, Demir, and Ingadottir, 2005).

In this study, all tested samples showed higher redness (a) and yellowness (b) than soy protein isolates. The values observed for protein isolates of body wall, flower and internal organs were 2.10±0.26, 2.42±0.15 and 2.97±0.07, respectively. The corresponding b values ranged from 17.85 to 18.64, having highest b value for internal organs and lowest for body wall protein isolates. Presence of pigments, blood and the amount of dark muscle in the samples may be attributed to the color of sea cucumber protein isolates (Surasani, 2018). In addition, physical parameters, including structure of the protein, presence of chromoproteins and amount of moisture in the sample, could have an influence in determining the color of the final product (Nolsøe and Undeland, 2009). It has been reported that the pH-shift processing method can remove chromoproteins from muscle

foods but it was less efficient in the pigment removal process (Surasani, 2018). For example, adjusting the pH will affect the heme protein structure by causing denaturation, unfolding and degradation (Nolsøe and Undeland, 2009). In the isoelectric point precipitation step, unfolded heme proteins get oxidized and produce brown color associated with increased yellowness (Surasani, 2018), whereas co-precipitation of heme proteins may have contributed to the improvement in redness of the protein isolates (Kristinsson et. al., 2005).

Table 4.3 The color of protein isolates from sea cucumber *C. frondosa* and soy

Sample	L	a	b
BWI	57.80 ± 0.36^{b}	2.10 ± 0.26^{b}	17.85 ± 0.10^{b}
FLI	43.01 ± 0.40^{c}	2.42 ± 0.15^{b}	18.01 ± 0.11^{a}
INI	41.91 ± 0.98^{c}	2.97 ± 0.07^{a}	18.64 ± 0.45^{a}
SPI	71.26 ± 0.46^{a}	1.32 ± 0.46^{c}	12.41 ± 0.46^{c}

L, whiteness; a, redness; b, yellowness; BWI, body wall protein isolate; FLI, flower protein isolate; INI, internal organs protein isolate; and SPI, soy protein isolate. Data are expressed as means ±SD from triplicate determinations.
Different superscripts in each column for the same color parameter of protein isolates indicate significant difference at $p< 0.05$.

4.4 Solubility of protein isolates

Protein solubility has a major influence on functional properties of protein products (Shahidi, 1994). The solubility curve of sea cucumber isolates and soy protein isolates as a function of pH is shown in Figure 4.1. All protein isolates exhibited a typical U-shaped curve where the lowest solubility value was displayed close to the isoelectric points of each protein isolate (pH range of 4-6). Protein solubility was sharply increased below and above the isoelectric point. These findings were similar to those reported for isolates prepared from skipjack tuna roe (Cha et al, 2020), sea cucumber (*Stichopus japonicus*) gut (Du et al., 2019), sea urchin gonad (Shang et al., 2020), skipjack tuna roe (Cha et al, 2020) and large yellow croaker fish (Du et al., 2020).

The solubility of proteins is mainly determined by their surface hydrophobicity and hydrophilicity (Kristinsson et. al., 2005). It is presumed that the hydrophobic and hydrophilic balance is dictated mainly by the net charge of protein molecules and electrostatic charges (Chavan, McKenzie and Shahidi, 2001; Gehring et al., 2011). Therefore, the drastic reduction of protein solubility at the isoelectric point is attributed to the zero net charge and decreasing electrostatic repulsion, promoting protein aggregation. In contrast, increased solubility at low and high pH levels is directly associated with the positive and negative charges of the protein isolates, leading to strong electrostatic repulsion between the molecules (Sahni et al., 2020).

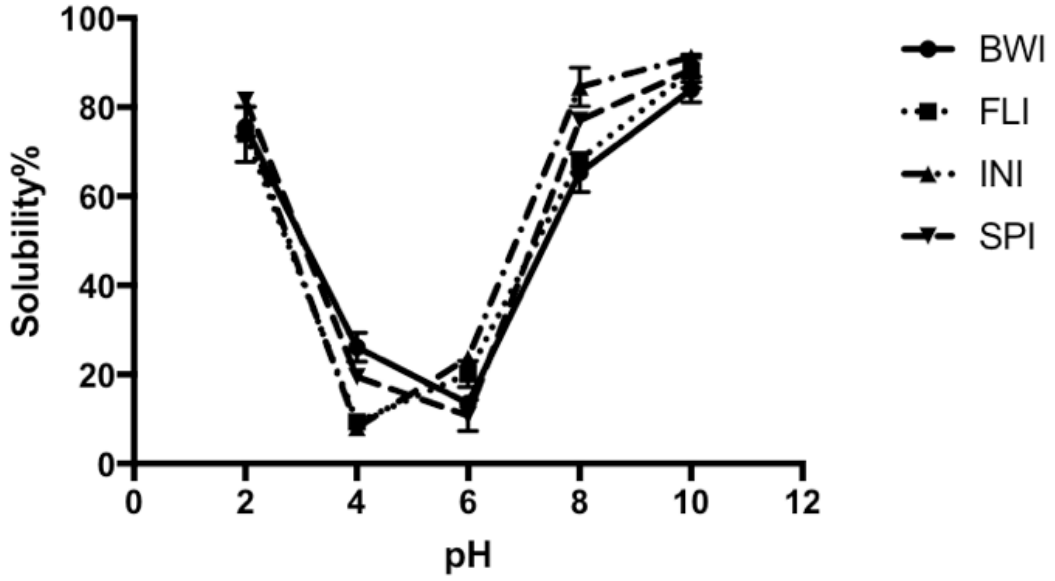


Figure 4.1 Solubility of sea cucumber *C. frondosa* protein isolates at various pH values BWI, body wall protein isolate; FLI, flower protein isolate; INI, internal organs protein isolate; and SPI, soy protein isolate. Values for each determination are means of three biological replicates.

4.5 Water holding capacity (WHC) and oil holding capacity (OHC)

Water holding capacity and oil holding capacity have direct relation with functional and sensory attributes of the final product, which affects the consumer perception of a food product (Gehring et al., 2011). WHC can be defined as the capacity of proteins to bind water against gravity and is considered to be one of the determinant factors of the texture, mouthfeel and viscosity of food products (Chavan et al., 2001; Abdollahi and Undeland, 2019). WHC of a protein is dictated by various factors, including protein structural factors such as conformation, hydrophobicity, as well as physical parameters like temperature and pH (Du et al., 2019). WHC of sea cucumber protein isolates (Figure 4.2) was within the range of 2.14±0.39 and 3.29 ±0.41 g/g protein, while soy protein isolates (SPI) showed significantly higher WHC of 10.2 ±0.30 g/g protein. These findings were in accordance

with the previously reported studies of WHC of protein isolates from large yellow croaker fish roe (Du et al., 2020). The highest WHC of sea cucumber samples was observed in flower protein isolates followed by body wall (2.28 ±0.15 g/g protein) and internal organs (3.29 ±0.41 g/g protein).

OHC is an important index that indicates the amount of oil absorbed by unit weight of protein (Shang et al., 2020). Oil binding mechanism involves capillary interaction and is associated with the retention ability of protein molecules (Zielińska, Karaś and Baraniak, 2018). Thus, OHC of protein may be attributed to the protein composition, surface area and hydrophobicity (Ghaly, Ramakrishnan, Brooks, Budge and Dave, 2013). Previous studies have revealed that proteins with low molecular weight and low density possess better oil holding capacity than proteins with high molecular weight and high density (Aryee, Agyei and Udenigwe, 2018). In addition, OHC is closely related to flavour retention, emulsification and shelf life (Deng et al., 2019). The highest OHC value was observed for body wall protein isolate (7.82±0.33 g/g protein) followed by these of flower (6.84±0.79 g/g protein) and internal organs (6.31±0.54 g/g protein). Soy protein isolates showed significantly (p<0.05) lower OHC than sea cucumber protein isolates. These results suggest that sea cucumber protein isolates could serve as a better alternative to soy protein isolate for enhancing the flavour and the texture of appropriate food products.

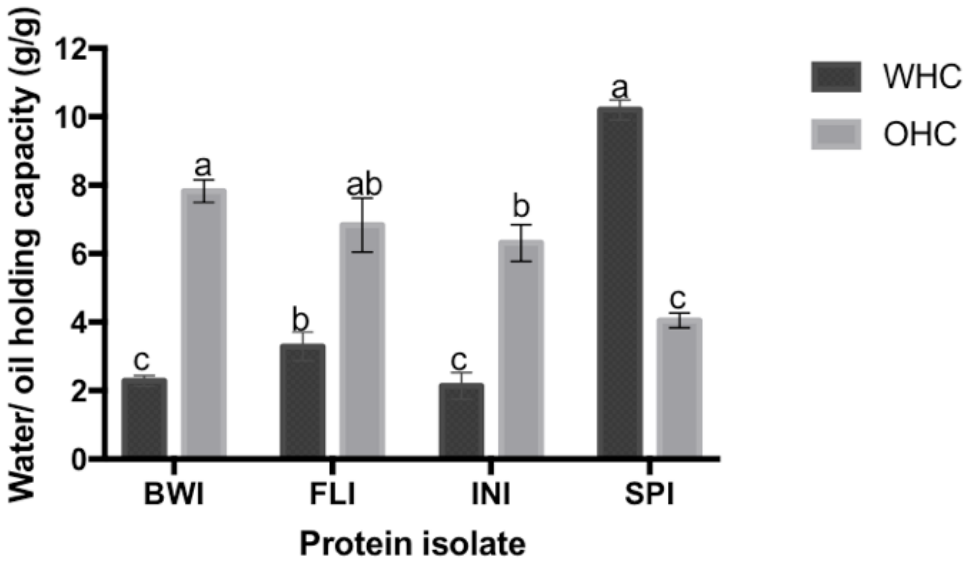


Figure 4.2 Water and oil holding capacities of sea cucumber *C. frondosa* protein isolates BWI, body wall protein isolate; FLI, flower protein isolate; INI, internal organs protein isolate; and SPI, soy protein isolate.

Different letters for the same property of protein isolates indicate significant difference at $p< 0.05$.

4.6 Emulsifying properties of sea cucumber protein isolates

Emulsifying properties can be assessed by emulsification activity index (EAI). These indices can characterize the ability of proteins to intermix and remain in an oil/ water interface (Shahidi et al., 1995; Du et al., 2020). EAI evaluates the area of oil-water interface stabilized by a unit weight of protein whereas emulsion stability is resisting breakdown, expressed as ESI (Taheri, Farvin, Jacobsen and Baron, 2014).

EAI and ESI for sea cucumber protein isolates were evaluated over the pH range of 2-10 with soy protein isolate used as a standard (Table 4.4). It has been reported that pH is one of the important determinant factors that affects the emulsification of a protein by changing its solubility (Du et al., 2020). EAI and ESI of sea cucumber isolates followed a similar trend to protein solubility. All protein isolates (body wall, flower, internal organs

and soy protein) exhibited the lowest EAI and ESI values at the pH range of 4-6. These results correspond to the solubility curve which indicates that pH values closer to the isoelectric point may increase strong interaction between protein molecules that block the diffusion-dependent migration of protein molecules at oil-water interface (Sahni et al., 2020). Thus, the adsorption of dissolved protein at the oil/water interface was insufficient to form emulsion. In addition to that, unavailability of proteins to unfold at the oil/water interface due to the poor charge distribution close to the isoelectric point is also another factor that affects the emulsification ability of proteins (Chavan et al., 2001). Proteins with low or few net chargers and weak electrostatic repulsion may facilitate the aggregation and precipitation of the molecules (Deng et al., 2019).

The observed emulsification properties of sea cucumber protein isolates exhibited a significant change when pH moved away from its isoelectric point. As pH increased (6-10 pH), the EAI of sea cucumber and soy protein isolates displayed the highest emulsion activity. A similar trend was reported in protein isolates from sea cucumber (*Stichopus japonicus*) guts (Du et al., 2018), scallop gonads (Han et al., 2019) and sea urchin gonads (Shang et al., 2020). The highest EAI values positively correlated with protein solubility. This observation may be associated with the strong electrostatic repulsion between the droplets, which prevents protein aggregation. For example, increasing of negative charges may increase the repulsion intensity, leading to the prevention of flocculation and coalescence of the protein molecules (Deng et al., 2019). Moreover, the high electrostatic charge of sea cucumber protein isolates can effectively influence the unfolding of protein structure and enhance emulsion stability (Du et al., 2020). The highest EAI was observed for flower protein isolate; but both body wall and internal organ protein isolates displayed

similar EAI to that of soy protein isolate at pH 10. A strong ESI was observed for both flower and body wall protein isolates compared to that of soy protein isolate. Therefore, higher EAI and ESI values of sea cucumber protein isolates were observed at pH values away from their isoelectric points. These findings support the fact that solubilized proteins have the potential to form a densely packed film around the oil droplet by adsorbing at the oil/water interface, promoting emulsification activity (Deng et al., 2019). The findings of the present study indicate that sea cucumber protein isolates prepared from body wall, flower and internal organs have the potential to be used as viable alternative emulsion stabilizer in selected foods.

Table 4.4 Emulsifying stability index (ESI) and emulsifying activity index (EAI) of sea cucumber *C. frondosa* and soy protein isolates

Sample	PH	ESI	EAI
BWI	2	12.19±0.71^{b}	62.65±0.81^{c}
	4	9.30±1.06^{c}	21.72±0.99^{e}
	6	13.16±0.48^{ab}	54.25±0.58^{d}
	8	15.08±1.08^{a}	74.52±0.63^{b}
	10	14.95±0.91^{a}	78.28±0.95^{a}
FLI	2	12.04±0.22^{c}	71.93±0.18^{c}
	4	9.18±0.26^{d}	33.40±0.67^{d}
	6	12.75±0.60^{c}	75.76±0.66^{b}
	8	16.89±0.48^{b}	77.29±0.94^{b}
	10	18.28±0.76^{a}	82.20±0.66^{a}
INI	2	10.07±0.64^{c}	66.66±0.56^{d}
	4	7.32±0.34^{d}	27.23±0.53^{e}
	6	11.15±0.59^{c}	63.74±0.65^{c}
	8	13.20±0.35^{b}	71.97±1.04^{b}
	10	16.57±0.28^{a}	78.57±0.92^{a}
SPI	2	12.83±0.48^{c}	64.75±1.12^{c}
	4	9.92±0.69^{d}	23.99±0.84^{d}
	6	13.63±0.83^{bc}	44.56±1.97^{b}
	8	14.77±0.65^{b}	72.32±1.34^{a}
	10	19.03±0.78^{a}	75.91±1.77^{a}

BWI, body wall protein isolate; FLI, flower protein isolate; INI, internal organs protein isolate; and SPI, soy protein isolate. Data are expressed as means±SD from triplicate determinations.

Different superscripts in each column of protein isolates indicate significant difference at $p< 0.05$.

4.7 Foaming properties of sea cucumber protein isolates

Foaming is an important physicochemical characteristic of a protein that relates to trapping of gas (air) bubbles in liquid (Nalinanon, Benjakul, Kishimura and Shahidi, 2011). Foam formation is governed by three main factors, namely transportation, penetration and reorganisation of molecules at the air-water interface (Zielińska et al., 2018). Therefore, it has been suggested that, during bubbling, proteins should rapidly adsorb at the air-liquid interface and undergo unfolding and rearrangement at the interface in order to exhibit a better foaming ability (Nalinanon et al., 2011). As shown in Table 4.5, the foaming capacity index (FCI) and foaming stability (FSI) of the sea cucumber protein isolates were pH-dependent and showed correspondence to their protein solubility. The foaming behaviour of proteins is mainly influenced by the pH of the dispersing media and extent of protein-protein interaction within the matrix (Klompong, Benjakul, Kantachote, Hayes and Shahidi, 2008). Maximum FCI and FSI values were observed at the pH range of 8-10 whereas lowest values were displayed closer to the isoelectric point of protein isolates. Compared to soy protein isolate, both body wall and flower protein isolates exhibited better foaming capacity, while internal organ protein isolate showed the lowest at the isoelectric point. These results indicate that improved solubility of proteins due to pH change can affect the dispersion's foaming capacity. Protein solubility promotes water-protein interactions, thus facilitating the unfolding of protein structure by allowing it to quickly spread at the air/water interface (Deng et al., 2019). The unfolded proteins enhance the stability of foams by reducing the surface tension of the liquid-air interface (Zielińska et al., 2018).

The lowest FCI values imply limited availability of proteins to diffuse at the air/ water interface. Protein aggregation at the isoelectric point due to low charge distribution and reduction of repulsive intensity leads to poor foam stability (Du et al., 2020). The maintenance of the stability of foam strongly correlates with surface adsorption, protein flexibility and surface hydrophobicity that affect the formation of cohesive interfacial membranes (Deng et al., 2019). The results of the present study are in accordance with the foaming capacity and foaming stability of protein isolates prepared from sea cucumber (*Stichopus japonicus*) guts (Du et al., 2018), sea urchin gonads (Shang et al., 2020), edible insects (Zielińska et al., 2018) and Chinese quince seeds (Deng et al., 2019). Therefore, the findings of this study suggest that sea cucumber protein isolates prepared from body wall, internal organs and flower have the potential to be used in a wide range of foods as functional ingredients with effective foaming properties.

Table 4.5 Foaming stability index (FSI) and foaming capacity index (FCI) of sea cucumber *C. frondosa* and soy protein isolates

Sample	pH	FSI	FCI
BWI	2	12.15±0.42^{c}	23.44±0.84^{d}
	4	6.89±0.69^{d}	17.50±1.08^{e}
	6	14.88±0.39^{b}	27.30±0.46^{c}
	8	15.92±0.66^{ab}	30.69±0.42^{b}
	10	17.37±0.50^{a}	36.04±0.71^{a}
FLI	2	13.22±0.51^{b}	27.12±0.70^{c}
	4	3.82±0.46^{d}	16.89±0.69^{d}
	6	7.96±0.34^{c}	27.89±0.69^{c}
	8	13.99±0.57^{b}	30.71±0.39^{b}
	10	17.78±0.51^{a}	32.91±0.63^{a}
INI	2	10.96±0.34^{b}	18.12±0.57^{c}
	4	3.36±0.42^{d}	11.99±0.66^{d}
	6	5.92±0.54^{c}	24.28±0.54^{b}
	8	11.08±0.63^{b}	27.86±0.64^{a}
	10	14.56±1.17^{a}	27.42±0.79^{a}
SPI	2	15.05±0.57^{b}	25.97±0.60^{c}
	4	4.10±0.59^{c}	18.88±0.68^{e}
	6	16.27±0.69^{ab}	22.97±2.13^{d}
	8	15.42±0.25^{b}	31.12±0.43^{b}
	10	17.87±0.87^{a}	34.89±0.69^{a}

BWI, body wall protein isolate; FLI, flower protein isolate; INI, internal organs protein isolate; and SPI, soy protein isolate.

Data are expressed as means±SD from triplicate determinations.

Different superscripts in each column for each protein isolate under same sample indicate significant difference at $p< 0.05$.

4.8 Surface hydrophobicity (S_o)

Surface hydrophobicity is mainly influenced by amino acid composition, processing conditions, and physical parameters such as pH (Deng et al., 2009). In particular, proteins isolated by pH shifting method exhibited increased surface hydrophobicity (Gehring et al., 2011). Extreme acidic or alkaline conditions could alter the native status of protein due to the presence of proton (H^+) or hydroxide ion (^-OH). Thus, S_o is considered as an indicator of protein conformation and correlates with functionality of proteins, including solubility and emulsifying properties (Xue et al., 2019). A high S_o indicates the possible exposure of hydrophobic regions existing in the protein molecules due to unfolding of protein structures (Deng et al., 2019). Particularly, exposing of hydrophobic amino acid residues, including tyrosine, phenylalanine, tryptophan, and valine, may also improve the surface hydrophobicity of protein molecules (Han et al., 2019).

The S_o of sea cucumber protein isolates was evaluated according to the method developed by Chelh et al. (2006), which is based on the interaction of the hydrophobic chromophore bromophenol blue (BPB) with proteins. The BPB method is considered as a simplified procedure for fluorescence probe method, widely used despite reports on the complications due to interference of other molecules in the biological system (Chelh et al., 2006). The BPB method is often used to determine the hydrophobicity of meat proteins and the amount of bound BPB is referred as the index for protein hydrophobicity (Xue et al., 2019). The hydrophobicity of sea cucumber protein isolates followed the order of body wall> flower> internal organs (Table 4.6). The sea cucumber protein isolates exhibited

significantly higher surface hydrophobicity than soy protein isolate. These observations may be attributed to the spatial conformation of the sea cucumber proteins and higher exposure of hydrophobic residues due to the denaturation during pH-shifting processing method.

4.9 Sulfhydryl (SH) group and disulfide (SS) bond measurements

Sulfhydryl groups and disulfide bonds play important roles in protein conformation by promoting structural stability and rigidity to the protein molecule (Deng et al., 2019). The changes in sulfhydryl groups and disulfide bonds in muscle proteins are crucial factors for quality parameters of muscle foods (Synowiecki and Shahidi, 1991). The highest amount of sulfhydryl (SH) groups and disulfide bonds among the sea cucumber isolates were observed in body wall followed by flower and internal organs (Table 4.6). Moreover, the free SH content was significantly higher in body wall while both flower and internal organ protein isolates had similar contents of free SH. It has been suggested that proteins with higher content of disulfide bonds may contribute to the stability of protein structure by tightening the peptide chains in protein molecules (Deng et al., 2019).

The total SH groups contain both bound and free SH groups. The observed total SH and free SH groups of the present study were lower than the reported values for protein isolates prepared from sea cucumber (*Stichopus japonicus*) guts (Du et al., 2018) and higher than the scallop gonads (Han et al., 2019). It is presumed that relatively low amounts of free-SH reflect the high extent of protein denaturation associated with the processing conditions (Han et al., 2019). For example, alkaline treatment may expose the thiol groups unfolding the protein structure (Shang et al., 2020). In addition, extreme acidic conditions

may contribute to the oxidation of SH groups, formation of SS and SH/SS interchange reactions during protein isolation process (Xue et al., 2019; Du et al., 2020; Yang et al., 2020). The sulfhydryl oxidation could cleave the disulfide bonds of proteins and release free SS groups (Han et al., 2019). Thus, decrease of SH groups in sea cucumber protein isolates groups compared to that of soy protein isolates may be associated with the processing conditions, including precipitation, solubilization and lyophilization.

Table 4.6 Surface hydrophobicity and sulfhydryl groups of sea cucumber *C. frondosa* and soy protein isolates

Parameter	Protein isolates			
	Body wall	Flower	Internal organs	Soy protein
Surface hydrophobicity (Bound BPB μg)	69.73±0.81^{a}	63.92±0.91^{b}	52.78±0.96^{c}	43.48±0.83^{d}
Free SH (μmol/g)	6.14±0.32^{a}	4.95±0.07^{b}	5.40±0.54^{ab}	6.22±0.03^{a}
SS (μmol/g)	34.82±0.57^{b}	33.10±0.38^{c}	30.81±0.27^{d}	40.53±0.50^{a}
Total SH (μmol/g)	75.78±0.98^{b}	71.15±0.82^{c}	67.02±0.62^{d}	87.28±1.05^{a}

SH, sulfhydryl groups; SS, disulfide bonds; BWI, body wall protein isolate; FLI, flower protein isolate; INI, internal organs protein isolate; SPI, soy protein isolate.
Data are expressed as means±SD from triplicate determinations.
Different superscripts in each column of protein isolates indicate significant difference at $p< 0.05$.

4.10 Structure analysis of protein isolates

Fourier transform infrared (FTIR) spectroscopy is a well-established technique for analyzing the secondary structure of protein (Senadheera et al., 2020). In general, FTIR spectra reflect the unique vibrations of the structural units of the protein related to their secondary structures (Tran et al., 2020). The IR absorption range of amide I, II and III is important specifically for the shape of the peptide. The amide I band is considered as an important marker of secondary structure of proteins (Zhong, Chen, Hu and Ren, 2015). The secondary structures of protein may include β-turn (1,660 to 1,700 cm^{-1}), α-helix and irregular structure (1,640 to 1,659 cm^{-1}), as well as β-sheet or extended structure (1,620 to 1,640 cm^{-1}) in amide I region (Muyonga, Cole and Duodu, 2004; Zhong et al., 2015). In addition, the carbonyl (C=O) stretching vibration of the peptide chain is present in the amide I range 1,700 to 1,600 cm^{-1}. Amide II (1550 to 1,480 cm^{-1}) is mainly associated with N-H bond and C-N expansion whereas of amide III (1,320 to 1,220 cm^{-1}) reflects the C-N stretching and N–H bending (Li et al., 2020; Sahni et al., 2020). In addition, amide A is important for the random coil determination which includes wave numbers between 3225 and 3280 cm^{-1} for hydrogen bonds (Tran et al., 2020).

The FTIR spectra of sea cucumber protein isolates are shown in Figure 4.3, 4.4, 4.5 and soy protein isolate in Figure 4.6. The major absorption bands were identified for protein structure are presented in Table 4.7. The secondary structure of sea cucumber protein isolates had similar peak positions for all the three tested samples that arose in the amide I region, including α-helix at the wave number of 1,539 cm^{-1} for body wall protein isolates and 1,541 cm^{-1} for flower and internal organ isolates. The same wave numbers were

attributed to the N-H bending vibrations of amide II region. Characteristic β-sheet peaks were observed at 1,637 cm^{-1} for body wall and internal organs whereas peak at 1,636 cm^{-1} represented the β-sheet for flower protein isolate. The CN stretching and N-H bending of amide III region were indicated by the wave number of 1,250 cm^{-1} for body wall and flower protein isolates and 1,252 cm^{-1} for internal organs. Furthermore, absence of anti-parallel intermolecular β-sheet peak (1,685 cm^{-1}) indicates that sea cucumber protein isolates may not be aggregated, however this needs to be confirmed using other experimental means. The distinct peak at amide A region of the FTIR spectra of body wall and flower was identical to that of soy protein (3,277 cm^{-1}). However, the absorption peak at 3,280 cm−1 was observed in internal organ protein isolates, which generally corresponds to the OH-vibration of phenolic compounds. This may be due to the presence of residual phenolic compounds in the medium. The formation of protein-phenolic complexes may directly influence the physicochemical properties of protein including solubility, hydrophobicity, emulsification and foaming abilities. In addition, protein-phenolics interactions may interfere with the availability of amino acid residues (Ambigaipalan and Shahidi, 2015). Thus, lower functional properties of internal organs may be due to the effect of protein-phenolic interactions.

However, overall FTIR spectra of sea cucumber isolates exhibited distinct absorption peaks at main wave numbers and our findings provide some information about the secondary structure of proteins in all three sea cucumber samples tested.

Table 4.7 Identification of characteristic peaks in IR spectrum of sea cucumber *C. frondosa* and soy protein isolates

Sample	Frequency (cm^{-1})	Characteristics IR absorption peaks for protein (cm^{-1})	Remarks
BWI	1637	Amide I region (1700-1600)	C=O stretching vibration
	1539	Amide II region (1550 - 1480)	N-H bond and C-N expansion
	1250	Amide III region (1320 - 1220)	C-N stretching and N–H bending vibrations
	3275	Amide A region (3280- 3,225)	H bonds
FLI	1636	Amide I region (1700-1600)	C=O stretching vibration
	1541	Amide II region (1550 - 1480)	N-H bond and C-N expansion
	1250	Amide III region (1320 - 1220)	C-N stretching and N–H bending vibrations
	3277	Amide A region (3280- 3225)	H bonds
INI	1637	Amide I region (1700-1600)	C=O stretching vibration
	1541	Amide II region (1550 - 1480)	N-H bond and C-N expansion
	1248	Amide III region (1320 -1220)	C-N stretching and N–H bending vibrations
	3280	Amide A region (3280- 3225)	H bonds; Characteristic peak 2924 and 3280 of OH vibrations
SPI	1637	Amide I region (1700-1600)	C=O stretching vibration
	1526	Amide II region (1550 -1480)	N-H bond and C-N expansion
	1252	Amide III region (1320 -1220)	C-N stretching and N–H bending vibrations
	3277	Amide A region (3280- 3225)	H bonds

BWI, body wall protein isolate; FLI, flower protein isolate; INI, internal organs protein isolate; and SPI, soy protein isolate.

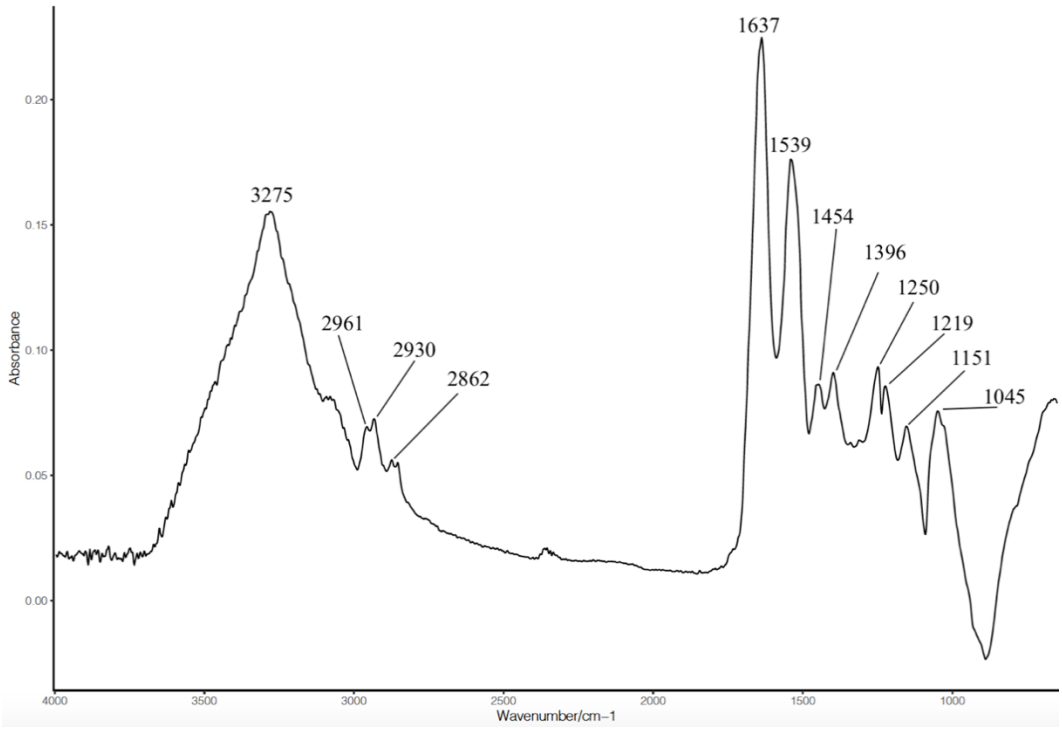


Figure 4.3 FTIR spectrum of body wall protein isolates

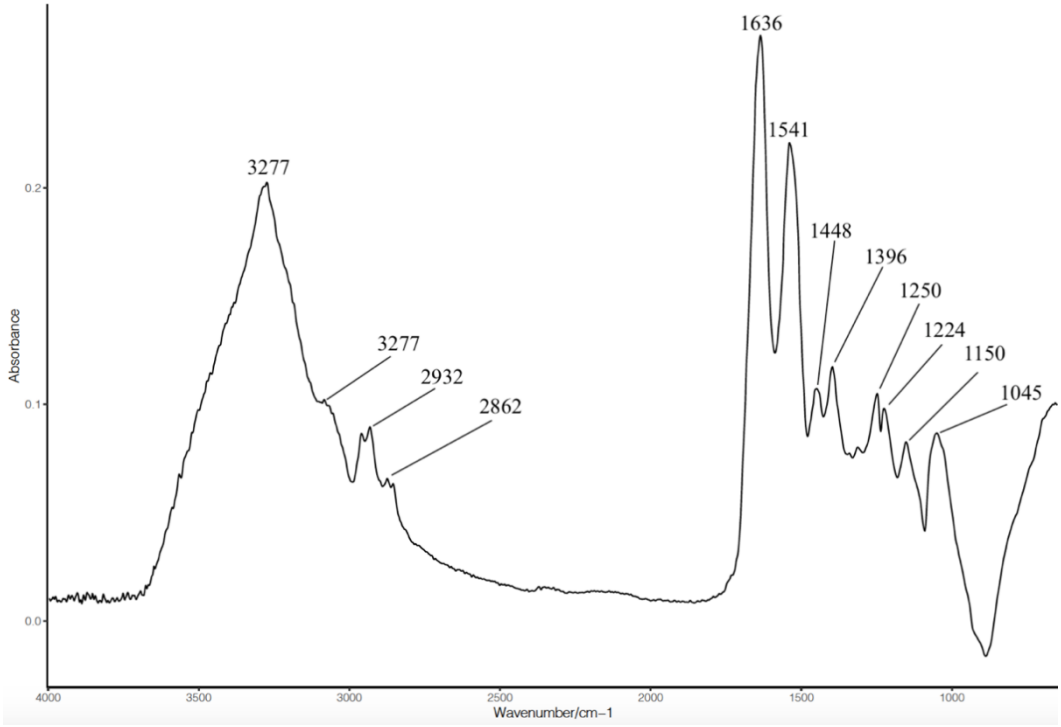


Figure 4.4 FTIR spectrum of flower protein isolates

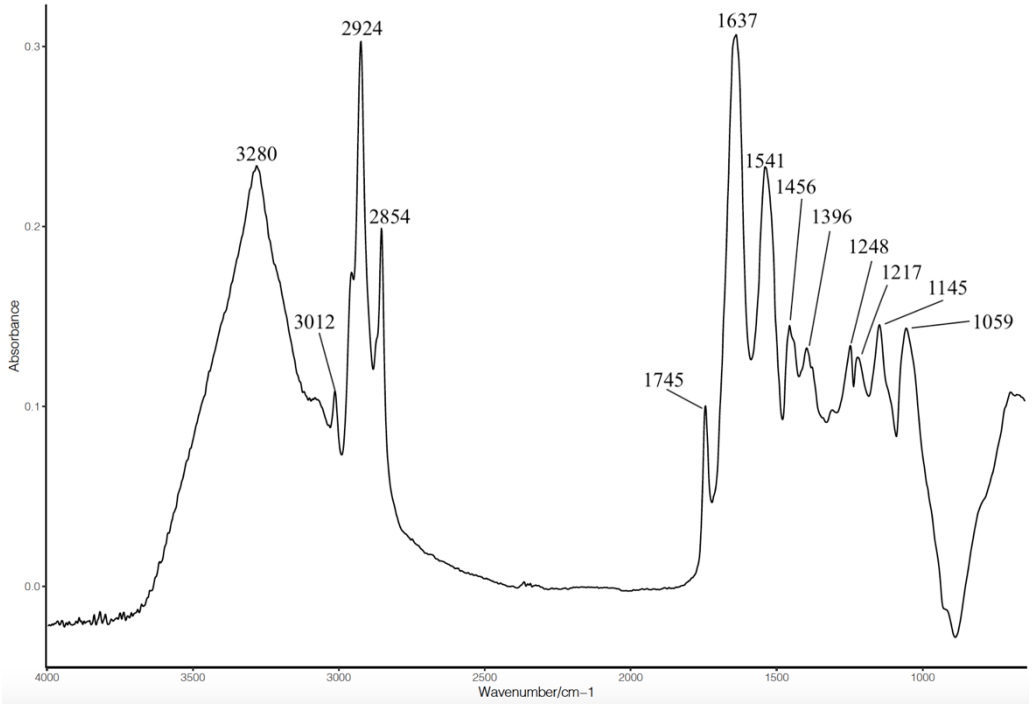


Figure 4.5 FTIR spectrum of internal organs protein isolates

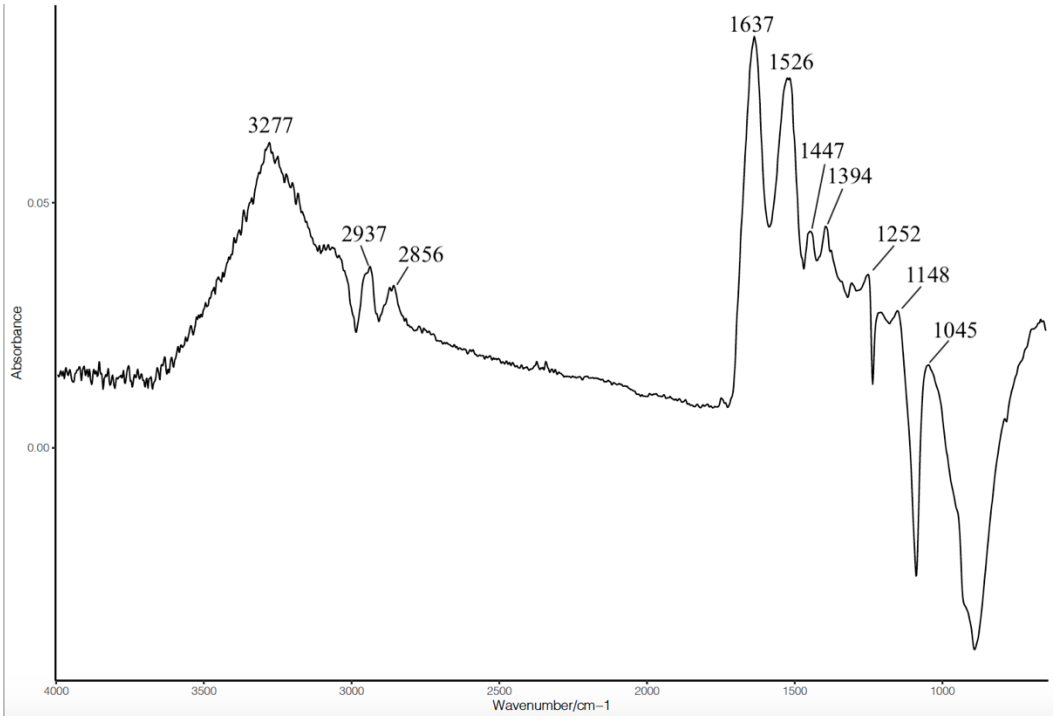


Figure 4.6 FTIR spectrum of soy protein isolates

4.11 Summary

Protein isolates recovered from body wall, flower and internal organs showed distinct functional properties compared to soy protein isolate. Compositional analysis of sea cucumber protein isolates demonstrated that they could be utilized as rich sources of essential amino acids and revealed their potential to use as food supplements. All recovered proteins showed better water and oil holding capacities, emulsifying and foaming properties compared to soy protein isolate. This indicates their potential to be used in a wide range of food applications. Findings of surface hydrophobicity and sulfhydryl group measurements may be attributed to the spatial conformation of the sea cucumber protein isolates associated with the exposure of hydrophobic residues during processing. Furthermore, FTIR spectra of the samples reflects that recovered protein isolates possessed unique absorption peaks related to the secondary structure of protein.

Thus, the results suggest that the pH-shift process can be used as an effective biorefinery approach for isolating high quality proteins from different body parts of sea cucumber.

CHAPTER 5

BIOACTIVE POTENTIALS AND PHYSICOCHEMICAL PROPERTIES OF SEA CUCUMBER PROTEIN HYDROLYSATES

5.1 Introduction

Marine by-products are rich protein sources for production of protein hydrolysates and peptides with unique bioactive properties (Shahidi, 1994). The most common means of producing protein hydrolysates in the industrial sector includes chemical and biological approaches (Kristinsson and Rasco, 2000). Production of protein hydrolysates using enzyme hydrolysis has been attracting increased attention as an effective method to produce functional food ingredients and nutraceuticals for disease risk reduction and health promotion (Ambigaipalan, Al-Khalifa and Shahidi, 2015). The most frequently used enzymes are derived from plants, animals and microbial sources (Lopez-Pedrouso et al., 2020). However, microbial proteases such as Alcalase, Flavourzyme, and Corolase are popular in industrial production due to their favourable operational conditions (Kristinsson and Rasco, 2000). In addition, enzymatic modification of proteins is mainly determined by the type of enzyme used in the hydrolysis process. Different types of proteinases, including endo- and exoproteinases are used to hydrolyze the peptide linkages. Endopeptidases hydrolyse the peptide bonds within polypeptide chains, while exopeptidases (aminopeptidase and carboxypeptidase) catalyze the cleavage of the terminal peptide bond from a polypeptide chain or a protein (Panyam and Kilara, 1996).

Functionality and the bioactivity of hydrolysed food proteins are due to the peptides that contain 2-20 amino acid units (Ambigaipalan and Shahidi, 2017). Molecular weight, amino acid sequence, hydrophobic and polar groups of the peptides determine the level of biological activity and functional properties of the hydrolysed product. A limited number of studies have reported the bioactive potential and functional properties of Atlantic sea cucumber by-products. These studies have been conducted on antioxidant activities (Mamelona et al., 2010b) and functional properties (Yan et al., 2016) from viscera as well as on antiviral activities from flower and internal organs (Tripoteau et al., 2015). Few of studies have been focused on the hydrolysis of whole animal to examine the potential of using hydrolysates as antiaging agents (Lin et al., 2018) and identification of antioxidative peptides (Zhang et al., 2020).

However, to date enzymatic hydrolysates of different tissues of *Cucumaria frondosa* have not been comparatively studied to assess their potential in controlling free radical attack on biological molecules such as lipids, proteins and ribonucleic acids as well as their chelating ability of prooxidant transition metal ions. Moreover, there is no report available on determining the effect of sequential use and individual endopeptidases and exopeptidases on the production of protein hydrolysates from Atlantic sea cucumber discards. Therefore, the current study for the first time investigates the effect of using Alcalase and Corolase as exopeptidases and Flavourzyme with both endopeptidase and exopeptidase activity were used for producing protein hydrolysates from different body parts of the Atlantic sea cucumber and reports their antioxidant potential in biological and food model systems.

5.2 Degree of hydrolysis (DH)

The degree of hydrolysis is an important parameter that can have a direct influence on biological function of peptides (Xie, Du, Shen, Wu and Lin, 2019). Defatted sea cucumber samples, hydrolysed with Alcalase (A), Flavourzyme (F) and Corolase (C), showed a similar pattern for degree of hydrolysis. Hydrolysis curves (Figure 5.1) show the DH (%) obtained for the hydrolysates produced from sea cucumber body wall (BW), flower (FL) and internal organs (IN) up to 4 h of hydrolysis. The increase in the hydrolysis of proteins was observed in BW, FL and IN for the first 2h. After 4h of hydrolysis with Alcalase, Flavourzyme and Corolase, DH values ranged between 6 and 13%, 5 and 8% in internal organs and 5 and 12% in body wall, flower and internal organs, respectively (Figure 5.2). The highest DH values in individual enzyme treatment were observed for Alcalase treated samples of each tissue (BWA~ 13.5%; INA~ 8.5%; FLA~12.5%). Nearly the same of DH was obtained for samples which were individually treated with Corolase and Flavourzyme in body wall hydrolysates (BWF ~9.5%; BWC~ 10.7%) after 4h of hydrolysis. A similar trend was observed in flower hydrolysates (FLF~11.7%; FLC~10.6%) and hydrolysates prepared from internal organs (INF~6.5%; INC~6.2%) during the same hydrolysis time. Similar findings were reported by Mamelona et al. (2010a) for Alcalase assisted hydrolysis of sea cucumber viscera (5.6%) and Yan et al. (2016). Under optimum hydrolysis conditions sea cucumber viscera was treated with Alcalase for 6h showed a higher DH value than that observed for Flavourzyme-assisted hydrolysis (Yan et al., 2016).

After an initial rapid phase, the rate of hydrolysis reached a stationary phase (Ambigaipalan and Shahidi, 2017). The rapid hydrolysis indicates that a large number of peptide bonds was hydrolyzed in the initial phase (Shahidi et al., 1995). The stationary phase may correlate with the decrease in hydrolysis sites, enzyme autodigestion or product inhibition (Khantaphant and Benjakul, 2008). Thus, increasing the concentration of specific enzymes may not be effective for obtaining a higher degree of hydrolysis. The typical hydrolysis curves were also reported for thronback ray fish (Lassoued et al., 2015), sardinella (Ben-Khaled et al., 2014) and zebra blenny (Ktari et al., 2012). Furthermore, protein hydrolysates with a degree of hydrolysis ranging from 1 to 10% have been reported to exhibit better functional properties than their precursor proteins (Panyam and Kilara, 1996; Ambigaipalan et al., 2015). Extensive hydrolysis of protein may be detrimental to the quality of final products due to the exposure and accumulation of hydrophobic side chains of amino acids which may lead to bitterness (Ambigaipalan et al., 2015). The observed values for the DH of sea cucumber protein hydrolysates were in the same range as those reported for Atlantic salmon muscle hydrolysates produced using Alcalase, Flavourzyme and Corolase (Kristinsson and Rasco, 2000; Liu et al., 2020) and shrimp hydrolysates produced with Alcalase and various other enzymes (Ambigaipalan and Shahidi, 2017).

Among the single enzyme treatments, Alcalase showed the highest degree of hydrolysis in all three sea cucumber body parts. Alcalase is widely used to produce functional protein hydrolysates from fish protein (Shahidi and Synowiecki, 1997; Kristinsson and Rasco, 2000; Thiansilakul et al., 2007; Klompong, et al., 2008). In addition,

Flavourzyme and Corolase have also shown excellent potential for hydrolysing food proteins (Neves et al., 2017).

During hydrolysis, major structural changes in proteins are linked with the breaking down of polypeptide chains into smaller peptide units (Klompong et al., 2008; Rao, Bajaj and Mann, 2020). In addition, use of a single enzyme for hydrolysis process shows relatively lower effectiveness than treatment with combinations of enzymes (Kristinsson and Rasco, 2000). Villanueva et al. (1999) reported that sequential use of endopeptidase and exopeptidases could render a higher degree of hydrolysis and generate hydrolysates with better functional properties compared to single enzyme treatment. Hydrolysates obtained from using endopeptidase and exopeptidase sequentially exhibited significantly higher ($p < 0.05$) DH values compared to that of single enzyme treated samples in all the three sea cucumber body parts. The observed DH values for combined Corolase and Flavourzyme treatment were 16, 11.8 and 17.3% for body wall, flower and internal organs, respectively. Reported DH values of the body wall, internal organs and flower hydrolysates prepared with combination of Alcalase and Flavourzyme were 18.3, 10.7 and 16.4%, respectively. The observed trend was previously reported in several studies indicating that single enzyme application is not effective in bringing about extensive hydrolysis of food proteins (Rao et al., 2020; Xu et al., 2020). Variations in DH values could be attributed to the difference in specificity of enzymes during hydrolysis (Lassoued et al., 2015)

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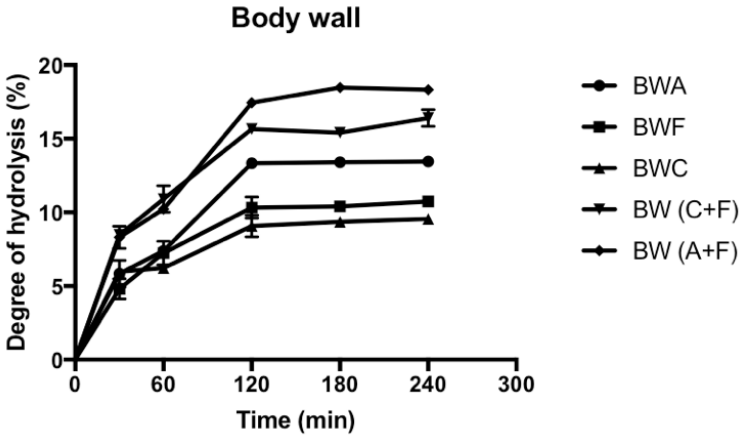

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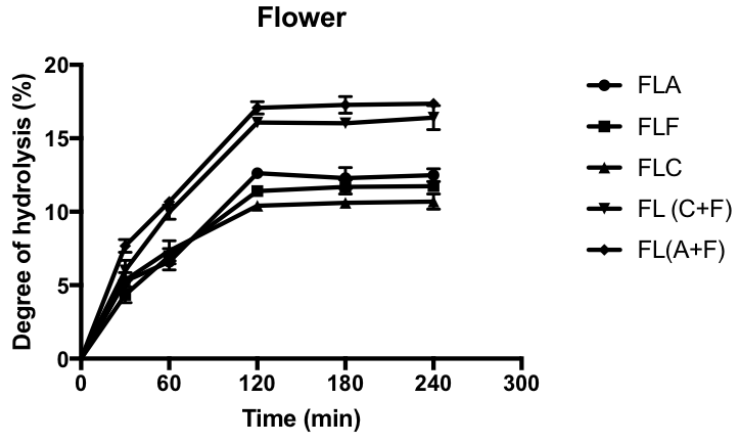

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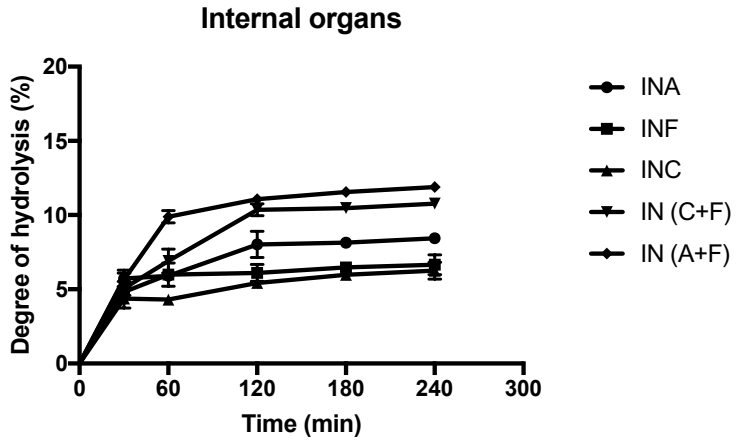


Figure 5.1 Degree of hydrolysis (DH) of sea cucumber *C. frondosa* protein hydrolysates a) BW, body wall; b) FL, flower; and c) IN, internal organs; A, Alcalase; F, Flavourzyme; C, Corolase; C+F, combination of Corolase and Flavourzyme; and A+F, combination of Alcalase and Flavourzyme.

5.3 DPPH radical scavenging activity

The DPPH radical is a commercially available stable chromogen radical and DPPH radical scavenging assay is one of the most frequently used methods in determining the antioxidant activity of food (Shahidi and Zhong, 2015). This assay is based on neutralization of DPPH radical by accepting a hydrogen atom or an electron from antioxidants. The colorimetric change of the purple color of DPPH into yellow is measured at 517 nm using UV spectrophotometry. In addition, efficacy of antioxidant can be monitored by electron paramagnetic resonance (EPR) spectroscopy (Shahidi and Yeo, 2020). The EPR assay monitors the intensity of the EPR signal when DPPH radical is scavenged by an antioxidant substance. When DPPH radical is scavenged by hydrolysates, it suggests that hydrolysates contain amino acids or peptides that are electron or hydrogen donors (Nalinanon et al., 2011). Furthermore, presence of hydrophobic amino acids in the hydrolysates makes the peptides more accessible to DPPH by increasing their solubility in a non-polar environment. This favours interaction of peptides with radicals and trap them in order to terminate the radical chain reaction (You, Zhao, Regenstein and Ren, 2010). The radical scavenging ability of food proteins and their hydrolysates depend mainly on the various factors such as size and the amino acid composition of the peptides, specificity of the protease and DPPH assay conditions (Girgih, Udenigwe and Aluko, 2011).

DPPH radical scavenging of protein hydrolysates prepared from body wall, flower and internal organs were in the range of 10-14, 12-16 and 7-12 µmol of trolox/ g of protein, respectively (Figure 5.2). Similar results were reported in the literature for protein hydrolysates from tilapia scale (7.56 µmol of trolox/ g of protein) (Ngo Qian, Ryu, Park and Kim, 2010), and shrimp shell protein hydrolysates (9.8-17.5 µmol of trolox/ g of

protein) (Ambigapalan and Shahidi, 2017). Moreover, DPPH radical scavenging activity of *C. frondosa* hydrolysates with different treatments exhibited different radical scavenging activities. However, as indicated in Figure 5.2, no significant difference (p>0.05) was observed in the internal organ hydrolysates treated with Flavourzyme, Corolase and two enzyme combinations. Similar trends were observed in the body wall and flower hydrolysates. In body wall, Flavourzyme treated samples and samples treated with combination of Alcalase and Flavourzyme exhibited no significant difference (p>0.05) in DPPH radical scavenging activities whereas flower hydrolysates prepared with Flavourzyme, Corolase and combined treatment of Corolase and Flavourzyme showed similar activity with no significant difference (p>0.05) in scavenging DPPH radicals. Furthermore, almost all samples treated with Flavourzyme or its combinations exhibited comparatively higher DPPH radical scavenging activity than other enzymes. Yan et al. (2016) suggested that hydrolysates prepared with Flavourzyme display high surface hydrophobicity and DPPH radical scavenging activity which is associated with the presence of hydrophobic amino acids or peptides. However, the difference in antioxidant activities among all treatments and reported values could be attributed to the existing differences in amino acid composition, sequence and conformation (Girgih et al., 2011).

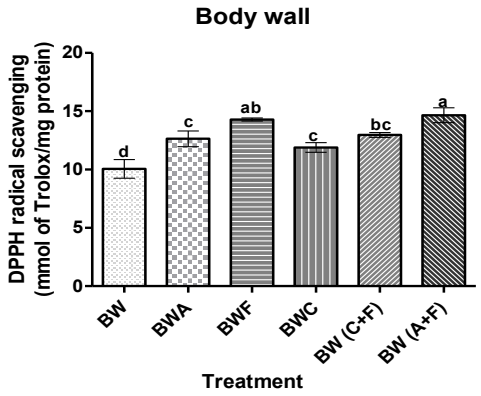
Flower hydrolysates prepared with the combination of Alcalase and Flavourzyme exhibited the highest DPPH radical scavenging activity among all treatments. Slizyte et al. (2016) reported similar findings for hydrolysates prepared from salmon backbones with combination of bromelain, papain and pepsin. They reported the highest DPPH radical activity in samples treated with a combination of enzymes compared to using single enzyme treatments. In addition, He, Wang, Ning, Yang and Wang, (2014) also revealed that

anchovy protein hydrolysates prepared from endogenous enzymes combined with commercial proteases exhibited higher antioxidant activity than single enzyme treated samples.

Furthermore, untreated counterparts of all the tested body parts of sea cucumber *C. frondosa*, showed significantly lower ($p< 0.05$) DPPH radical scavenging activities compared to the samples hydrolysed with Alcalase, Flavourzyme and Corolase. These findings are in accordance with the radical scavenging activities reported by Yan et al. (2016) for enzyme hydrolysates prepared from viscera of *C. frondosa*. The authors suggested that hydrolysates prepared with alkaline proteases such as Alcalase and Flavourzyme possess higher antioxidant activities than other tested enzymes including Neutrase, papain, bromelain and pepsin. They concluded that alkaline proteases are preferred for generating bioactive hydrolysates from sea cucumber samples (Yan et al., 2016).

Studies have shown that use of different enzymes for hydrolysing food proteins leads to varying antioxidant potentials in final products (Cumby et al., 2008). Furthermore, antioxidant capacity of each protein hydrolysate depends on its composition. For example, composition of Alcalase assisted protein hydrolysates from flower may be completely different from that of Flavourzyme hydrolysates. This may account the varied antioxidant activities observed among the different enzyme treated sea cucumber hydrolysates.

[a]



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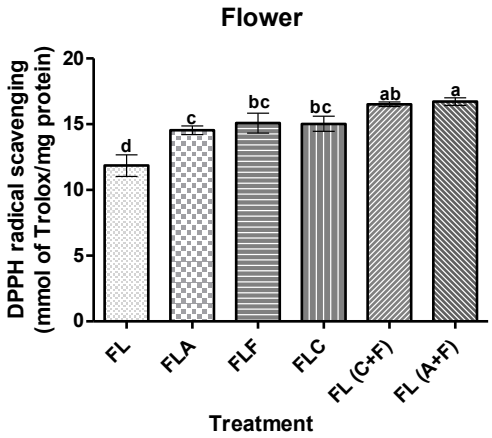

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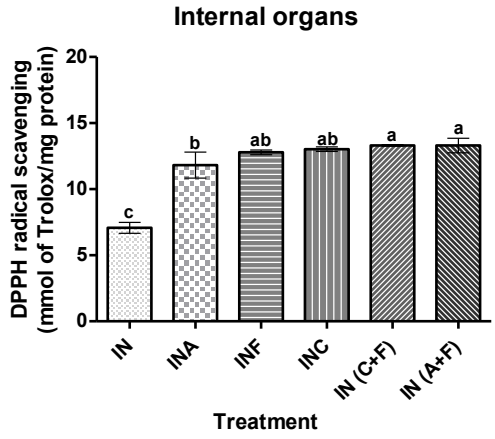


Figure 5.2 DPPH radical scavenging activity of [a] BW, body wall; [b] FL, flower; [c] IN, internal organs and of sea cucumber *C. frondosa* (A, Alcalase; F, Flavourzyme; C, Corolase; A+F, combination of Alcalase and Flavourzyme; C+F, combination of Corolase and Flavourzyme). Different letters for the same concentration of all hydrolysates indicate significant difference at $p < 0.05$.

5.4 ABTS radical scavenging assay

The ABTS assay measures the ability of antioxidants to scavenge the stable radical cation ABTS•+, the blue/ green chromophore which has a maximum absorbance at 734 nm (Shahidi and Zhong, 2015). The chemical reaction between the strong oxidizing agent potassium persulfate produces the $ABTS^{\bullet+}$ that can be neutralized by direct reduction or by radical quenching (Shahidi and Yeo, 2020). Hence, this assay is based on single electron transfer (SET) as well as hydrogen atom transfer (HAT) mechanisms which can be used to determine both lipophilic and hydrophilic molecules (Ambigaipalan et al., 2015). It is assumed that hydrophilic antioxidative compounds show high ABTS radical-scavenging activity (Nalinanon et al., 2011). In general, hydrolysates consist of hydrogen donating peptides or proteins that have the ability to react with radical cations and terminate the radical chain reaction (Khantaphant and Benjakul, 2008).

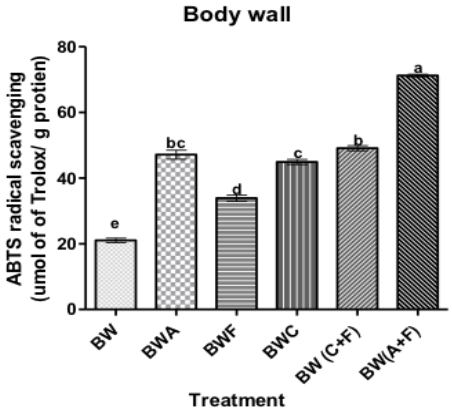
The ABTS radical scavenging activity of protein hydrolysates produced from different body parts of the sea cucumber showed significantly (p <0.05) higher values than their untreated counterparts (Figure 5.3). Hydrolysates prepared with flower using sequential enzyme treatments of Alcalase and Flavourzyme exhibited the highest ABTS radical scavenging activity (79± 0.55 µmol of trolox/ g of protein) among all treated samples while the lowest activity (17± 0.81µmol of trolox/ g of protein) was reported in the untreated internal organs sample. When considering overall ABTS radical scavenging activity of treated samples, protein hydrolysates produced from internal organs showed no significant difference (p >0.05) between samples treated with individual enzymes and enzyme combinations. The ABTS radical scavenging activity of sea cucumber samples

were similar to those of shrimp shell protein hydrolysates (Ambigaipalan and Shahidi, 2017). A similar trend was observed in both ABTS and DPPH radical scavenging activity with respect to the body wall and flower hydrolysates prepared by sequential treatment with Alcalase and Flavourzyme. The difference between the hydrolysates produced by different enzyme treatments dictate the antioxidant potential of the final products (Cumby et al., 2008).

These findings indicate that in addition to the degree of hydrolysis, free amino acids present after the hydrolysis process, amino acid sequence of the resultant peptides and their molecular weight could influence the radical scavenging activity of peptides (Intarasirisawat, Benjakul, Visessanguan and Wu 2012; Ambigaipalan et al., 2015). For instance, strong radical scavenging activities have been reported for tripeptides with tryptophan or tyrosine at their C-terminus. Moreover, difference in antioxidant potential of each sample may correlate with the different combinations of amino acids in the hydrolysed product (Saito et al., 2003). The difference in amino acid combinations of the resultant products is correlated with the hydrolysis of the polypeptide in various portions (Cumby et al., 2008). Hydrolysis patterns of Alcalase, Corolase and Flavourzyme are different; Alcalase and Corolase both act as endopeptidases whereas Flavourzyme has both exo- and endopeptidase activities (Kristinsson and Rasco, 2000). Therefore, Alcalase and Corolase produce small and medium size peptides. Even though, Alcalase and Corolase have endopeptidase activity, the principal functional group in their active site is different as Alcalase belongs to serine proteases and Corolase is a metalloprotease (Kristinsson and Rasco, 2000; Cumby et al., 2008). Moreover, Flavourzyme may produce amino acids and more low-molecular-weight peptides compared to endopeptidase (Hamada, 2000). Hence,

the varied antioxidant activities among different enzyme treatments may be due to their compositional difference. Therefore, it was assumed that sequential use of Alcalase and Flavourzyme may contribute to the generation of favourable compositional characteristics towards radical scavenging activities compared to other enzymes used in this study.

[a]



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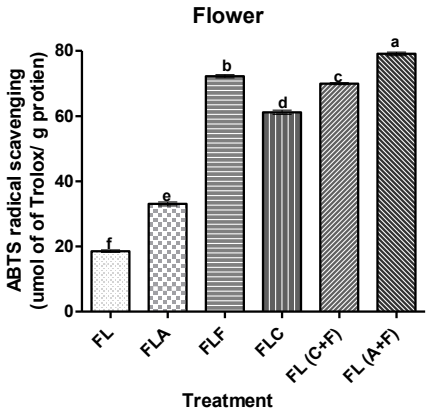

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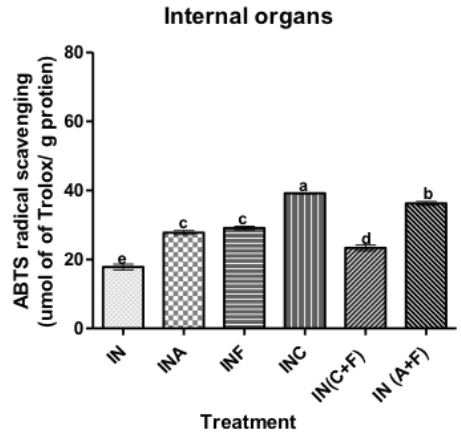


Figure 5.3 ABTS radical scavenging activity of sea cucumber *C. frondosa* protein hydrolysates a) BW, body wall; b) FL, flower; and c) IN, internal organs; A, Alcalase; F, Flavourzyme; C, Corolase; C+F, combination of Corolase and Flavourzyme; and A+F, combination of Alcalase and Flavourzyme. Different letters for the same concentration of all hydrolysates indicate significant difference at $p < 0.05$.

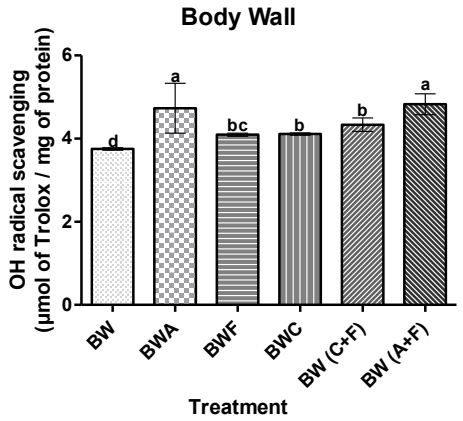
5.5 Hydroxyl radical scavenging assay

The hydroxyl radical is an oxygen derived radical which is considered as the most reactive free radical in biological systems. It can easily react with biomolecules such as amino acids, proteins DNA and membrane lipids (Xie et al., 2019). The excessive production of hydroxyl radicals may induce cellular damage through oxidative stress (Shahidi and Yeo, 2020). Thus, removal of the excessive levels of hydroxyl radical is considered as one of the effective defense strategies in preventing the occurrence of numerous cellular disorders such as cancer, cardiovascular and diabetes, among others (You et al., 2010). Hydroxyl radicals are generated in the presence of metal ions via Fenton reaction. The scavenging ability of antioxidant substances can be determined using EPR with the detection of the spin adduct of 5,5-dimethyl-1-pyrroline N-oxide (DMPO) (Je, Park and Kim, 2005). Hydroxyl radical scavenging activity of sea cucumber protein hydrolysates varied between 2.7 and 4.8µmol trolox equivalents / g of protein; a significant difference ($p > 0.05$) was observed between the enzyme treated and untreated samples of each sea cucumber body part (Figure 5.4). Hydrolysates prepared from flower with sequential enzyme treatment of Alcalase and Flavourzyme showed higher hydroxyl radical scavenging activities compared to all other enzyme treatments in each group. The hydroxyl radical scavenging activity in terms of percentage ranged from 11 to 27%. Similar observations were made by Zhang et al. (2020) on hydrolysates prepared from Atlantic sea cucumber using Alcalase and Trypsin. The authors suggested that enzyme treatments were responsible for improved antioxidant potential of protein hydrolysates compared to their untreated counterparts. However, as reported in both DPPH and ABTS radical scavenging

activities, there was no observable trend in internal organ hydrolysates in both single enzyme- and combined enzyme-assisted hydrolysis treatments.

Yan et al. (2016) stated that protein hydrolysates may scavenge hydroxyl radicals by electron/ hydrogen donation and radical quenching. In addition, Girgih et al. (2015) suggested that presence of hydrophobic amino acids inversely correlates with the hydroxyl radical activity. The authors reported that unfractionated protein hydrolysates exhibit a higher hydroxyl radical scavenging activity than fractionated peptides obtained from cod protein hydrolysates. These results indicated that fractionation of peptides may lead to the loss of their synergistic effect towards neutralizing free radicals. In contrast, peptide fractions prepared from hempseed proteins showed higher hydroxyl radical scavenging activity than their corresponding unfractionated hydrolysates (Girgih et al., 2011). However, Cumby et al. (2008) stated that radical scavenging activity of peptides or protein hydrolysates corelates with the hydrogen donor activity of the hydroxyl groups of aromatic amino acid residues (tyrosine, histidine, tryptophan and phenylalanine). Radical scavenging activity of these aromatic amino acid residues improves through resonance stabilization. Hence, presence and absence of such amino acids in peptides as well as their positioning in peptide sequence also influences antioxidant activity (Ambigaipalan and Shahidi, 2017). For instance, proper positioning of certain amino acids in the peptide sequence can improve their radical scavenging activities (Rajapaksha, Mendis, Jung, Je and Kim, 2005).

[a]



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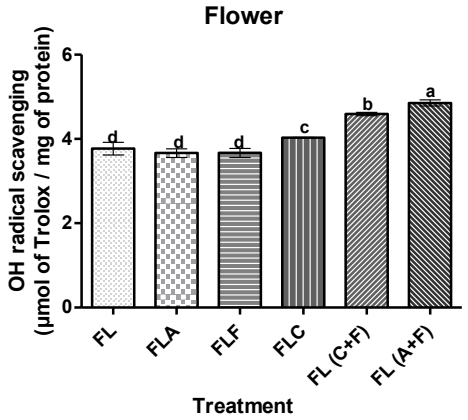

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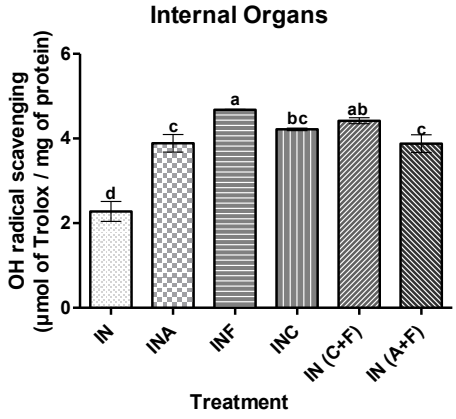


Figure 5.4 Hydroxyl radical scavenging activity of protein hydrolysates of [a] BW, body wall; [b]. FL, flower; [c] IN, internal organs of sea cucumber *C. frondosa* (A, Alcalase; F, Flavourzyme; C, Corolase; A+F, combination of Alcalase and Flavourzyme; C+F, combination of Corolase and Flavourzyme). Different letters on same concentration of all hydrolysates indicates significant difference at $p < 0.05$.

5.6 Reducing power assay

The ferric reducing antioxidant power assay is often used to determine the ability of natural antioxidants to donate electron or hydrogen atom (Yan et al., 2016). This assay is categorized under the single electron transfer (SET)-based method that involves in reducing higher valency elements to their lower valence state (Shahid and Zhong, 2015). Reduction of ferric ion (Fe^{3+})- ligand complex to the ferrous (Fe^{2+}) complex is monitored using the absorbance at 700 nm (Antolovich, Prenzler, Patsalides, McDonald and Robards, 2002). Previous studies have demonstrated correlation between reducing power and antioxidant activity of protein hydrolysates (Girgih et al., 2011). Most nonenzymatic antioxidant activities are mediated by redox reactions, including reducing power. Antioxidative peptides in protein hydrolysates have the ability to reduce Fe^{3+} /ferric cyanide complex to ferrous form.

The reducing power was varied from 0.12±0.02 to 0.48±0.00, 0.08±0.02 to 0.35±0.02 and 0.16±0.03 to 0.53±0.02 μmol of trolox per mg of proteins in body wall, internal organs and flower hydrolysates, respectively (Figure 5.5). Samples treated with a combination of Alcalase and Flavourzyme showed significantly higher ($p < 0.05$) reducing power in all three body parts of sea cucumber (body wall 0.48±0.00, flower 0.53±0.02, internal organs 0.35±0.02 μmol of trolox per mg protein) compared to other treated and their untreated counterparts. These results were in agreement with those of ABTS and DPPH radical scavenging activities. The trend for sea cucumber samples hydrolysed with Alcalase and Flavourzyme combination on reducing power was similar to that observed for radical scavenging activity of tested sample. The current results are supported by the

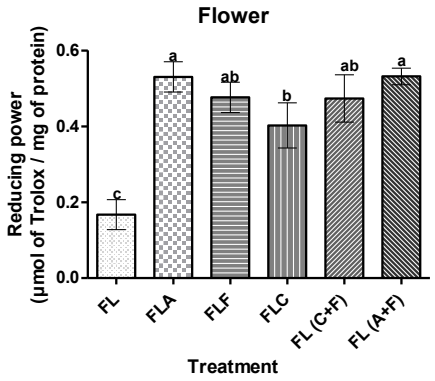
findings of Wiriyaphan, Chitsomboon and Yongsawadigul (2012) and Chalamaiah Jyothirmayi, Diwan and Kumar, (2015) who indicated that ferric reducing antioxidant power was directly influenced by the type of protease used for hydrolysis.

Yan et al. (2016) reported that reducing power of sea cucumber viscera was also influenced by the specificity of enzyme employed. The results indicated that samples hydrolyzed with Alacalase, Flavourzyme and trypsin possessed greater reducing power than those prepared by using bromelain, pepsin and papain treated samples. The authors suggested that the differences of the activity may be attributed to the presence of hydrophobic amino acids or peptides that can react with free radicals to form more stable products. Cumby et al. (2008) further explained this by suggesting that reducing power and other radical scavenging abilities of protein hydrolysates are composition dependent and may vary depending on the protease employed in the hydrolysis process.

The strong reducing power of protein hydrolysates is associated with the increased availability of hydrogen atom and electrons due to liberation of the peptides during the hydrolysis process (Chalamaiah et al., 2015). These peptides prevent the propagation of radical chain reactions.

According to Udenigwe et al. (2016), electron donation by amino acid residues including sulfhydryl group of cysteine also contributes to the reducing capacity of peptides. Therefore, presence of sulfhydryl group or their oxidized forms have direct impact on the reducing capacity of protein hydrolysates.

[a]



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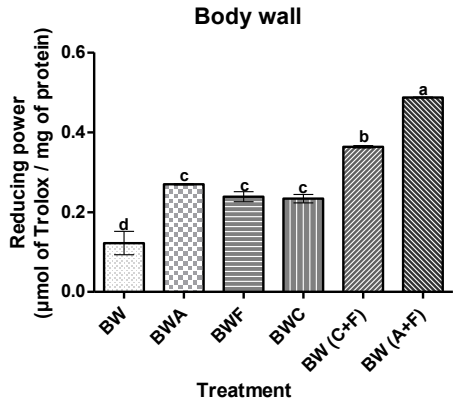

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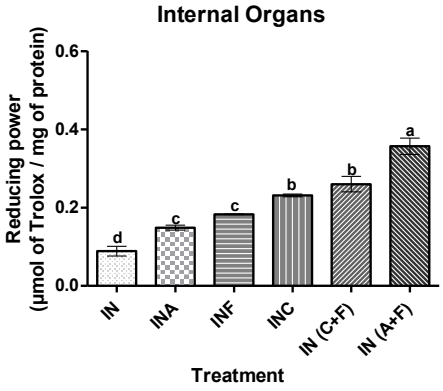


Figure 5.5 Reducing power of protein hydrolysates of [a] BW, Body wall; [b]. FL, Flower; [c] IN, internal organs of sea cucumber *C. frondosa* (A, Alcalase; F, Flavourzyme; C, Corolase; A+F, combination of Alcalase and Flavourzyme; C+F, combination of Corolase and Flavourzyme).Different letters on same concentration of all hydrolysates indicate significant difference at $p < 0.05$.

5.7 Metal chelation activity

Transition metal ions, specifically iron, participate in Fenton reaction, hence stimulate lipid oxidation. They can act as prooxidants and decompose hydroperoxide into volatile compounds (Klompong, Benjakul, Kantachote and Shahidi, 2007). In general, antioxidants with metal chelation ability can form complexes with prooxidant metal ions and making them unavailable for participating in lipid oxidation (Shahidi and Zhong, 2015). Metal chelation activity can be determined by measuring the loss of intensity of ferrozine-ferrous ion complex at 562 nm after addition of antioxidants. In general, peptides can form complexes with transition metal ions and retard the oxidation process (Girgh et al., 2015). This ability is associated with the presence of amino acid residues such as histidine, cystine, tryptophan, aspartate and glutamate that are known to bind divalent metal ions (Udenigwe et al., 2016).

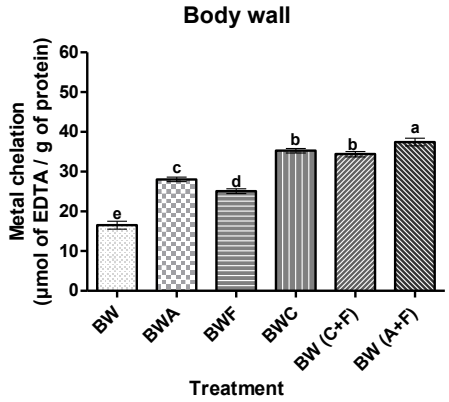
In this study, metal chelation ability varied from 16.5±0.90 to 37.43±0.98, 18.89±0.55 to 36.80±0.59 and 18.20±0.17 to 36.52±0.59 μmol of EDTA equivalents per g of protein in the hydrolysates prepared from body wall, internal organs and flower, respectively. Chelation of metal ions by sea cucumber protein hydrolysates showed that samples treated with combination of enzymes (Alcalase+Flavourzyme, Corolase+Flavourzyme) exhibited higher metal chelating ability compared to their untreated counterparts and single enzyme treated samples (Figure 5.6). The lowest metal chelation activity was noted in the untreated samples of each body part. Therefore, similar to radical scavenging abilities (DPPH, ABTS and hydroxyl) and the reducing power, metal chelation ability of the protein hydrolysates also varied according to the type of protease

used for hydrolysis. Interestingly, we observed that compared to radical scavenging activities and reducing power capacity, protein hydrolysates prepared from body wall, internal organs and flower using Corolase exhibited significantly higher ($p < 0.05$) metal chelation ability than the other single enzyme treatments. This may correlate with the hydrolysis mechanism of the Corolase as it belongs to the metalloprotease group of enzymes. Slizyte et al. (2016) stated that larger peptides have better ability to chelate the metal ions and their ability weakens with the progress of hydrolysis process. As depicted in Figure 5.6, metal chelation ability of hydrolysates produced from internal organs showed similar activity compared to the hydrolysates produced from body wall and flower. However, in radical scavenging activity assays and reducing power activity assay, internal organ hydrolysates exhibited weaker activity compared to the other two types of hydrolysates. These observations may have an association with protein configuration, amino acid sequence and composition of each protein hydrolysate that may be attributed to metal binding abilities. Slizyte et al. (2016) further explained that conflicting results of radical scavenging activities and metal chelation activities of the same hydrolysates can be expected due to the different protein properties related to different antioxidant activity mechanisms.

For instance, the differences observed between the samples may be due to having different charged amino acid side chain residues that have the ability to remove transition metal ions (Ambigaipalan and Shahidi, 2017). In addition, presence of histidine at the N terminal can be attributed to a strong metal ion chelation due its imidazole group. Furthermore, Girgih et al. (2015) stated that unfractionated protein hydrolysates exhibited better metal chelation activity than their fractionated counterparts due to the synergistic

effect of the component peptides. However, overall observations of the present study suggest that through an effective use of enzymes sea cucumber hydrolysates may enhance the chelation activity of prooxidant metal ions.

[a]



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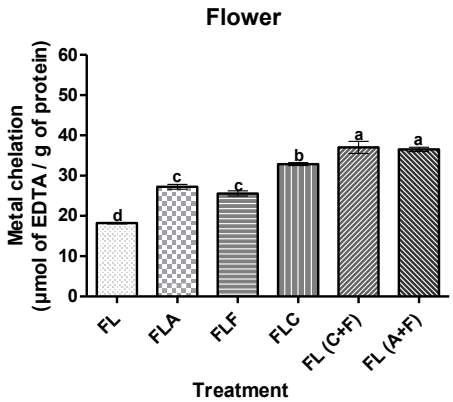

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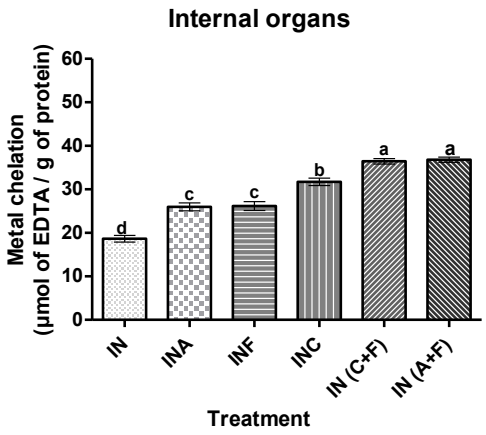

Figure 5.6 Metal chelation ability of protein hydrolysates of sea cucumber *C. frondosa* protein hydrolysates a) BW, Body wall; b) FL, Flower; and c) IN, Internal organs; A, Alcalase; F, Flavourzyme; C, Corolase; C+F, combination of Corolase and Flavourzyme; and A+F, combination of Alcalase and Flavourzyme. Different letters for the same concentration of all hydrolysates indicates significant difference at $p < 0.05$

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5.8 Antioxidant activity of protein hydrolysates in oil-in water emulsion system

The β-carotene bleaching assay is a classical method for evaluating the antioxidant activity in oil-in-water emulsion. Oxidation of linoleic acid in the emulsion system is monitored spectrophotometrically in the presence and absence of an antioxidant (Shahidi and Zhong, 2015) and simulates oxidation of membrane lipids (Nasri et al., 2013). Moreover, many food lipids which are prone to oxidation can be found as oil-in-water emulsions (Shahidi and Zhong, 2015). Thermally induced oxidation of linoleic acid forms free radicals and hydroperoxides in the emulsion system mainly by losing a hydrogen atom (Chandrasekara and Shahidi, 2010). The so formed free radicals attack β-carotene, a lipophilic chromophore (yellow-orange color) and this leads to decoloration as a result of losing its conjugation. The color change can be monitored with the time at 470 nm (Ambigaipalan and Shahidi, 2015). β-carotene is widely used as a coloring agent for food and beverages and its discoloration indicates the quality deterioration of the product (Sakanaka, Tachibana and Okada, 2005).The presence of antioxidants attenuates the rate of discoloration of β-carotene in the emulsion by scavenging the free radicals (Nasri et al., 2013).

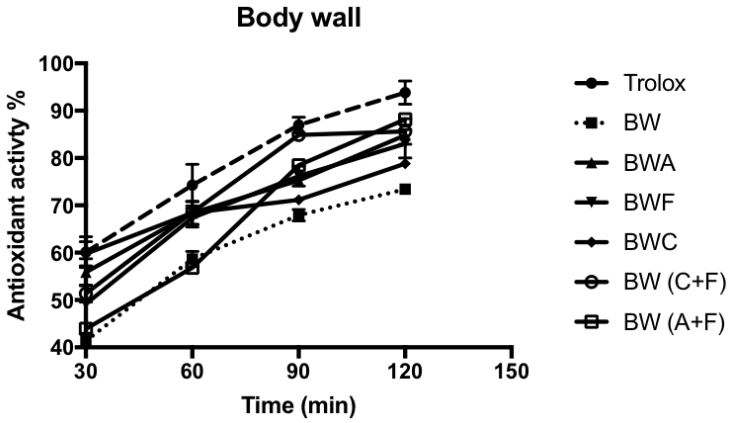
In this study, the enzyme treated samples showed a better inhibition of oxidation compared to their untreated counterparts (Figure 5.7). Rapid decoloration of β-carotene was observed in the control sample compared to all tested samples, whereas trolox which was used as the standard antioxidant exhibited the highest inhibitory activity. Overall, among the sea cucumber protein hydrolysates, samples prepared with combination of Alcalase and Flavourzyme showed significantly higher (p<0.05) inhibitory activity against

the decoloration of β-carotene. The observed inhibitory activity of protein hydrolysates and trolox up to 120 min ranged from 56 to 90%, including 88% in body wall (Alcalase+Flavourzyme), 86% in flower (Alcalase+Flavourzyme) and 68% in internal organs (Alcalase +Flavourzyme) of sea cucumber *C.frondosa*.

Similar observations were reported for protein hydrolysates prepared from capelin (Amarowicz and Shahidi, 1997), date seeds (Ambigaipalan and Shahidi, 2015) and shrimp shell discards (Ambigaipalan and Shahidi, 2017). Hydrophobic amino acids possess higher efficiency of preventing the oxidation in an oil-in water emulsion system compared to hydrophilic residues. This could be due to the orientation of hydrophobic amino acids at the oil-water interface (Ambigaipalan and Shahidi, 2017). Especially, hydrophobic amino acids have high affinity for linoleic acid. Therefore, it is presumed that peptides with hydrophobic amino acids could have the ability to scavenge lipid-derived radicals by donating H atom (Kim, Je and Kim, 2007). In addition, Kong and Xiong (2006) also reported that short-chain peptides have the ability to disperse at the water-oil interface in a liposome system and then be adsorbed to the phospholipid membrane in the liposome, where oxidation takes place.

According to Amarowicz and Shahidi (1997), discrepancy in antioxidant activities of peptides might be due to their synergetic action with emulsifier (Tween 40). Moreover, Nasari et al. (2013) have suggested that the β-carotene decoloration inhibitory activity of protein hydrolysates is influenced by the molecular size of the peptides, its chemical properties and electron transfer ability of its constituent amino acids.

[a]



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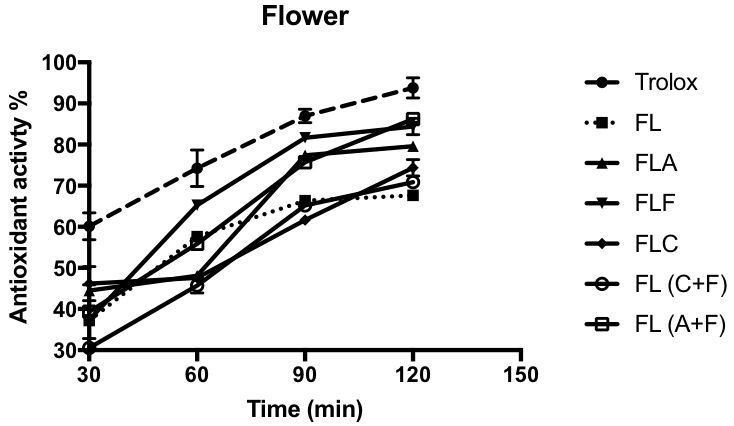

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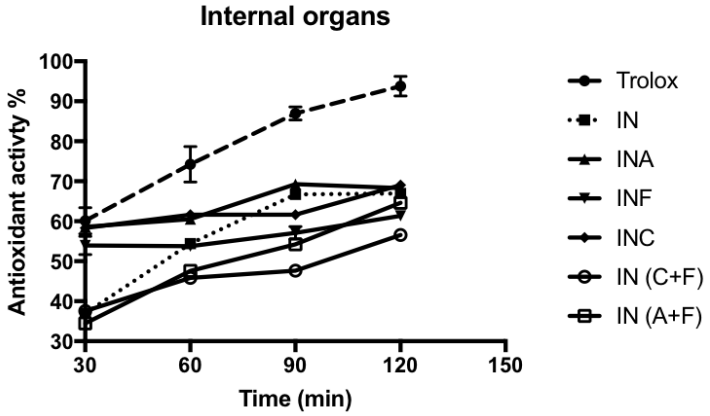


Figure 5.7 Antioxidant activity of sea cucumber *C. frondosa* protein hydrolysates in oil-in-water emulsion system. a) BW, Body wall; b) FL, Flower; and c) IN, Internal organs; A, Alcalase; F, Flavourzyme; C, Corolase; C+F, combination of Corolase and Flavourzyme; and A+F, combination of Alcalase and Flavourzyme. Different letters for the same concentration of all hydrolysates indicate significant difference at $p < 0.05$.

5.9 Antioxidant activity of sea cucumber protein hydrolysates in cooked comminuted meat model system (thiobarbituric acid reactive substances, TBARS)

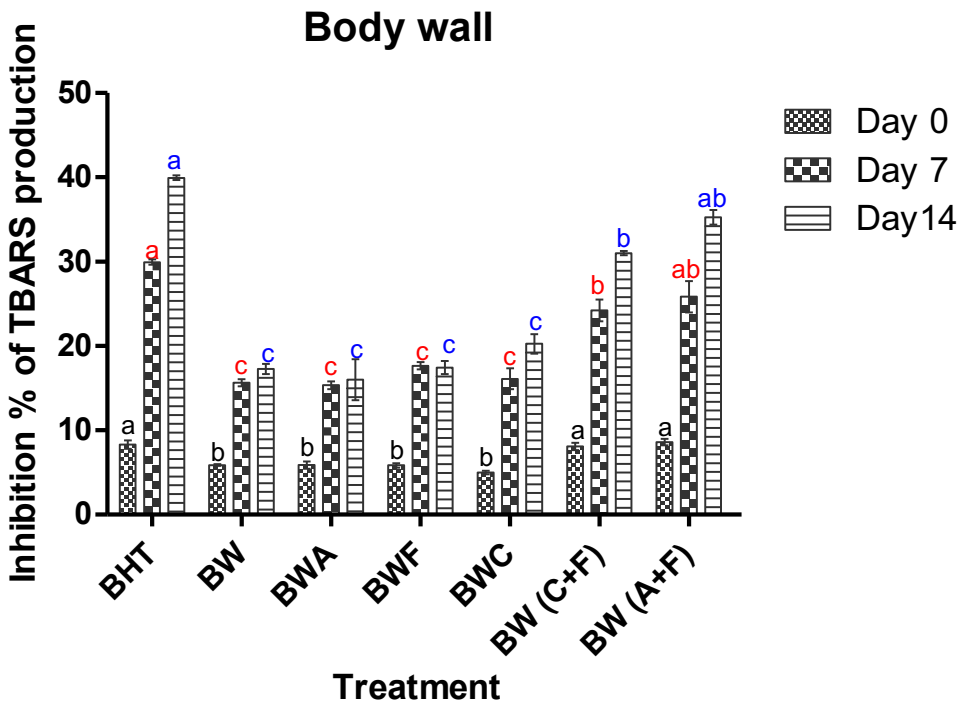
The thiobarbituric acid (TBA) assay was employed to assess the antioxidant efficacy of sea cucumber protein hydrolysates in a meat model system. TBA assay is a spectrophotometric detection method to determine the secondary lipid peroxidative product MDA (malonaldehyde) and related compounds. The secondary oxidative products include aldehydes and ketones and these are involved in quality deterioration of muscle foods (Shahidi and Zhong, 2015). The TBA reagent reacts with the MDA present in the sample to form an MDA-TBA adduct which absorbed at 532 nm (Shahidi et al., 1997). TBARS production in meat model systems incorporated with sea cucumber protein hydrolysates displayed significantly higher ($p < 0.05$) inhibitory activity compared to their untreated counterparts (Figure 5.8). Among all samples of each body part of sea cucumber hydrolysed with the combination of Alcalase and Flavourzyme exhibited the highest percentage of inhibition (body wall 25%, flower 28%, and internal organs 25%) followed by hydrolysates prepared with Corolase and Flavourzyme combination (body wall 24%, flower 22% and internal organs 13%) compared to BHT (29%) after seven days of storage. Moreover, the same trend was observed in samples treated with a combination of Alcalase and Flavourzyme after 14 days of storage in body wall (35%) and flower (36%) compared to the standard butylated hydroxytoluene (BHT) (39%) whereas internal organs (21%) showed slight decrease compared to its 7 days of storage period. However, the increase in TBARS correlates with production of low-molecular-weight oxidation products with progression of storage time. Further oxidation of short-chain products of lipid oxidation

which are unstable may also occur upon prolonged storage. Thus, some of the compounds so formed upon oxidation, such as acids and alcohols, are not determined by the TBA test (Kittiphattanabawon, Benjakul, Visessanguan and Shahidi 2012).

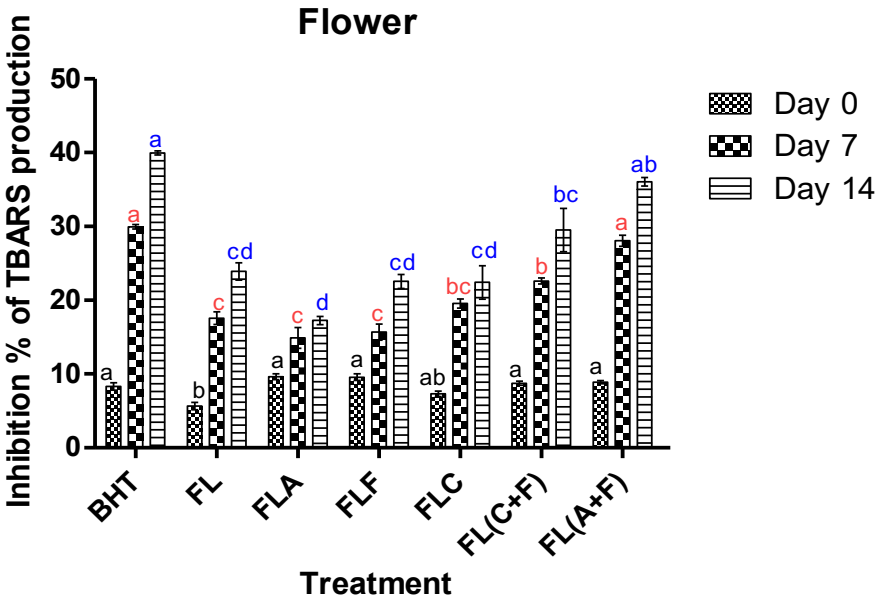
These observations are in accordance with inhibition of TBARS production of date seed protein hydrolysates (Ambigaipalan and Shahidi, 2015) and egg yolk protein hydrolysates (Sakanaka and Tachibana, 2006). The use of single enzyme (individual treatment of exopeptidase or endopeptidase) found to have lower inhibition of TBARS compared to the combined effect of both type of proteases. These findings indicated that protein hydrolysates prepared from sea cucumber using a combination of endopeptidases and exopeptidases could enhance the radical scavenging abilities and metal chelating activity that can have a direct impact on TBARS inhibition. Moreover, these findings of the inhibition of TBARS production in meat system by protein hydrolysates are in accordance with the radical scavenging activities (Figure 5.8) and metal chelation activities (Figure 5.6) of the current study. Hence, both *in-vitro* chemical assays and model system assays demonstrated a similar trend in evaluating antioxidant potential of sea cucumber protein hydrolysates.

Therefore, the results so obtained suggest that sea cucumber protein hydrolysates prepared using a combination of Alcalase and Flavourzyme as well as Corolase and Flavourzyme might be used as potential natural antioxidants in muscle foods.

[a]


Body wall
Inhibition % of TBARS production
50
40
30
20
10
0
Day 0
Day 7
Day14
BHT
BW
BWA
BWF
BWC
BW (C+F)
BW (A+F)
Treatment

[b]


Flower
Inhibition % of TBARS production
50
40
30
20
10
0
Day 0
Day 7
Day 14
BHT
FL
FLA
FLF
FLC
FL(C+F)
FL(A+F)
Treatment

[c]

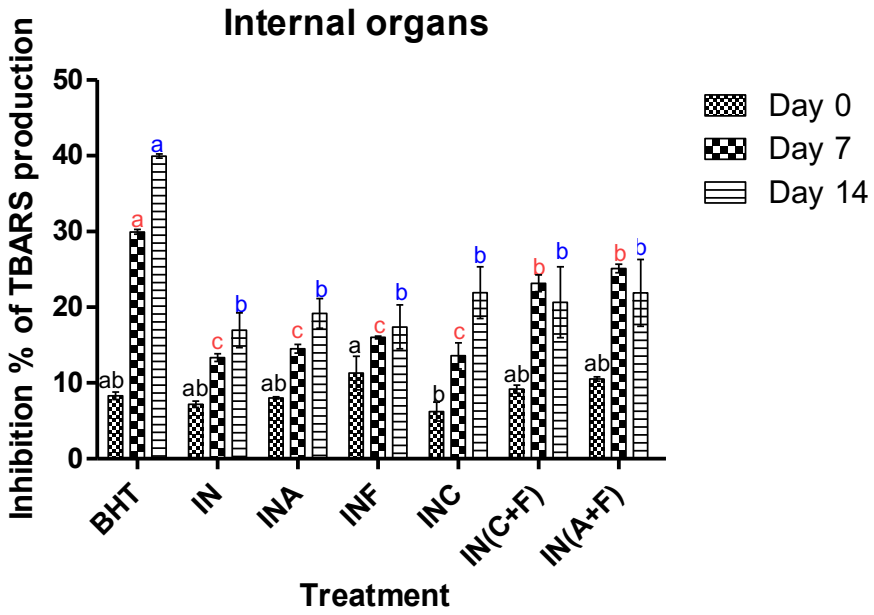


Figure 5.8 Inhibition % of TBARS production of protein hydrolysates of sea cucumber *C. frondosa* protein hydrolysates a) BW, body wall; b) FL, flower; and c) IN, internal organs; A, Alcalase; F, Flavourzyme; C, Corolase; C+F, combination of Corolase and Flavourzyme; and A+F, combination of Alcalase and Flavourzyme. Different letters for the same concentration of all hydrolysates indicate significant difference at $p < 0.05$.

5.10 Inhibition of hydroxyl and peroxyl radical-induced supercoiled DNA strand scission

Irreversible modification of DNA due to the oxidative damage may lead to mutation, carcinogenesis and other pathological processes (Shahidi and Yeo, 2020). Free radicals generated in living cells mediate the base modification, production of base free sites, DNA strand breakage, abnormal chromosomal arrangements and DNA-protein cross-links, among others (Chandrasekara, Daugelaite and Shahidi, 2018). Most reactive free radicals such as hydroxyl and peroxyl radicals possess a greater reduction potential and hence can react with biomolecules including damaging DNA at both the phosphate backbone and the nucleotide bases. For example, hydroxyl radical can abstract a hydrogen atom from pyrimidine and purine bases as well as from the deoxyribose sugar moieties of DNA (Ambigaipalan and Shahidi, 2015). Thus, it is crucial to supress DNA oxidation to avoid killing of the living cells. The current assay determines the inhibitory activity of antioxidants against hydroxyl and peroxyl and radical induced DNA strand scission in supercoiled plasmid PBR322 (Shahidi and Yeo, 2020). The supercoiled DNA (form I) could alter its conformation to nicked open circular form (form II) and a linear form (form III) as a consequence of oxidation of DNA induced by free radicals (Ambigaipalan and Shahidi, 2017). The inhibitory activity of an antioxidant against DNA strand scission is evaluated by considering the level of intact DNA strand and nicked DNA fractions using agarose gel electrophoresis (Shahidi and Zhong 2015). In general, a linear form of DNA showed restricted movements through the agarose gel network compared to the supercoiled DNA.

Sea cucumber protein hydrolysates were assessed for their protective effect against hydroxyl and peroxyl radical-induced strand scission. Carnosine, a β-alanylhistidine dipeptide is used as a positive control due to its physiological relevance as a natural antioxidant in muscle proteins. It is present in skeletal muscles at millimolar concentrations (Ambigaipalan and Shahidi, 2015). However, our preliminary experiments revealed that untreated protein counterparts of sea cucumber were unable to show any observable inhibitory affect against hydroxyl radical and peroxyl radical at a concertation of 6 mg/mL and 0.1mg/mL, respectively. All the treated samples showed significantly (p <0.05) higher inhibitory activity against peroxyl and hydroxyl radical induced DNA strand scission compared to carnosine (Figure 5.9).

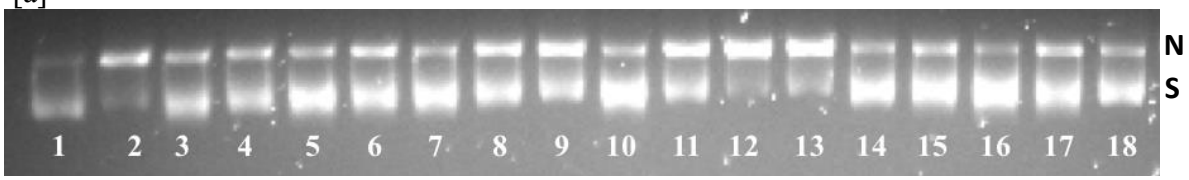
In these experiments, no clear trends were observed between single enzyme treated (exopeptidase or endopeptidase) and combined enzyme treated sea cucumber protein hydrolysates in inhibiting both hydroxyl and peroxyl radical mediated DNA strand scission. The inhibition of DNA scission induced by hydroxyl radical ranged from 30 to77% in body wall (Table 5.1,5.2 and 5.3), 35 to 49% in internal organ (Table 5.2) and 37 to 78% (Table 5.3) in flower hydrolysates. Moreover, inhibitory effect of peroxyl radical induced DNA oxidation was varied from 69 to 86% in body wall, 32 to 57% in internal organs and 27 to 89% in flower hydrolysates. Similar observations were reported by Ambigaipalan and Shahidi (2015) for date seed protein hydrolysates where the retention of supercoiled DNA was 13-33 and 47-83% for hydroxyl and peroxyl radical effects, respectively. These findings were also in accordance with the inhibitory activity of supercoiled DNA oxidation

indicated for blacktip shark gelatin hydrolysates (Kittiphattanabawon, Benjakul, Visessanguan and Shahidi, 2013).

The differences between reported values for hydroxyl and peroxyl radical activities could be due to the half-life of each radical. Hydroxyl radical is an extremely reactive free radical with shorter half – life compared to peroxyl radical which has relatively long half - life and has a high affinity to diffuse into cells (Kittiphattanabawon et al., 2013). Thus, the protective effect of sea cucumber protein hydrolysates was possibly due to the chelation of metal ions and scavenging activity of hydrolysates. Moreover, Lassoued et al. (2015) stated that DNA strand breakage could be due to the hydrophobic peptides. The inhibitory activity of hydrophobic scavengers on preventing plasmid DNA damage is more effective than hydrophilic scavengers (Yang and Schiah, 1996).

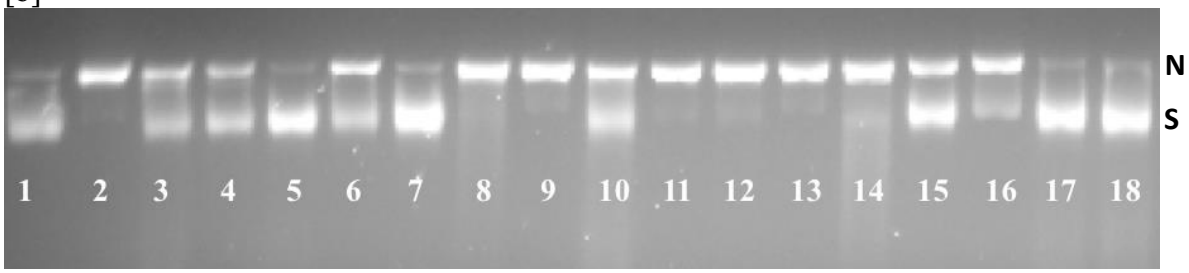
Kittiphattanabawon et al. (2013) suggested that biological activities of protein hydrolysates are determined by several factors including their constituent amino acids, peptides and their sequence, size and configuration, among others.

[a]



N- Nicked DNA; S-Supercoiled DNA; Control -DNA+ hydroxyl radical

[b]



N- Nicked DNA; S-Supercoiled DNA; Control -DNA+ peroxyl radical

1-Blank; 2-Control; 3-BWA;4- BWF;5- BWC;6- BW (C+F); 7- BW (A+F); 8-INA; 9-INF; 10-INC;11- IN(C+F);12- IN(A+F);13-FLA; 14-FLF;15-FLC;16-FL(C+F);17-FL (A+F);
18-Carnosine

Figure 5.9 [a] Agarose gel electrophoresis of inhibition of hydroxyl-radical induced DNA scission [b] peroxyl radical-induced DNA scission by hydrolysates of body wall (BW), internal organs (IN) and flower (FL) of sea cucumber *C. frondosa* (A, Alcalase; F, Flavourzyme; C, Corolase; A+F-Mixture of Alcalase and Flavourzyme; C+F- Mixture of Corolase and Flavourzyme)

Table 5.1 Inhibition of hydroxyl and peroxyl radical induced DNA scission by hydrolysates from sea cucumber *C. frondosa* body wall[1]

Sample	DNA Scission Inhibition (%)	
	Hydroxyl Radical	Peroxyl Radical
BWA	29.61±0.75^{c}	56.11±1.24^{c}
BWF	72.18±2.95 ^{ab}	69.45±3.90^{bc}
BWC	69.09±1.18^{b}	71.19±2.89^{b}
BW (C+F)	68.61±2.01^{b}	72.51±2.63^{b}
BW (A+F)	77.22±2.59^{a}	81.85±0.18^{a}
Carnosine	77.32±1.42^{a}	83.01±0.56^{a}

Abbreviations are: BW, body wall ; A, Alcalase; F, Flavourzyme; C, Corolase; A+F, combination of Alcalase and Flavourzyme; and C+F, combination of Corolase and Flavourzyme.

[1]All data represent the mean of triplicate determinations ± standard deviation. Values with the same letters in each column are not significantly different ($p > 0.05$) from one another.

Table 5.2 Inhibition of hydroxyl and peroxyl radical induced DNA scission by hydrolysates from sea cucumber *C. frondosa* flower[1]

Sample	DNA Scission Inhibition (%)	
	Hydroxyl Radical	Peroxyl Radical
FLA	38.59±2.27^{d}	57.41±3.53^{d}
FLF	37.64±2.69^{d}	63.06±2.47^{c}
FLC	69.24±0.51^{b}	72.67±1.32^{b}
FL (C+F)	72.58±0.65^{ab}	77.31±0.25^{ab}
FL (A+F)	76.94±1.09^{a}	79.79±1.36^{a}
Carnosine	77.32±1.42^{a}	83.01±0.56^{a}

Abbreviations are: FL, flower; A, Alcalase; F, Flavourzyme; C, Corolase; A+F, combination of Alcalase and Flavourzyme; and C+F, combination of Corolase and Flavourzyme.

[1]All data represent the mean of triplicate determinations ± standard deviation. Values with the same letters in each column are not significantly different ($p > 0.05$) from one another.

Table 5.3 Inhibition of hydroxyl and peroxyl radical induced DNA scission by hydrolysates from sea cucumber *C. frondosa* internal organs[1]

Sample	DNA Scission Inhibition (%)	
	Hydroxyl Radical	Peroxyl Radical
INA	35.77±1.55^{d}	57.14±2.64^{b}
INF	44.48±2.38^{c}	32.90±1.10^{d}
INC	58.49±2.50^{b}	61.53±3.16^{b}
IN (C+F)	46.71±3.27^{c}	53. 67±1.47^{bc}
IN (A+F)	49.49±0.95^{c}	47.28±2.02^{c}
Carnosine	77.32±1.42^{a}	83.01±0.56^{a}

Abbreviations are: IN, internal organ; A, Alcalase; F, Flavourzyme; C, Corolase; A+F, combination of Alcalase and Flavourzyme; and C+F, combination of Corolase and Flavourzyme.

[1]All data represent the mean of triplicate determinations ± standard deviation. Values with the same letters in each column are not significantly different ($p > 0.05$) from one another.

5.11 Inhibition of cupric ion- induced human low-density lipoprotein (LDL) peroxidation

Plasma low-density lipoprotein (LDL) oxidation, caused by the action of metal ions or reactive oxygen species, is considered one of the major risk factors for the development of atherosclerosis which is the primary cause of a majority of cardiovascular diseases (Chandrasekara and Shahidi, 2011; Pan et al., 2019). Therefore, inhibition of LDL oxidation can provide an effective strategy in preventing cardiovascular diseases

(Ambigaipalan and Shahidi, 2015). The copper-induced LDL oxidation assay determines the oxidative susceptibility of LDL by monitoring the formation of conjugated dienes (CD), the initiation phase of LDL oxidation (Rahman, Ambigaipalan and Shahidi, 2018). The increase in LDL oxidation is indicated by the change in the absorbance of CD at 234 nm. Increase in CD is linked to the formation of cholesteryl linoleate hydroperoxides and cholesteryl linoleate hydroxides (Kittiphattanabawon et al., 2013). Thus, the presence of both compounds may create the favourable condition for lipid peroxidation (Ambigaipalan and Shahidi, 2015).

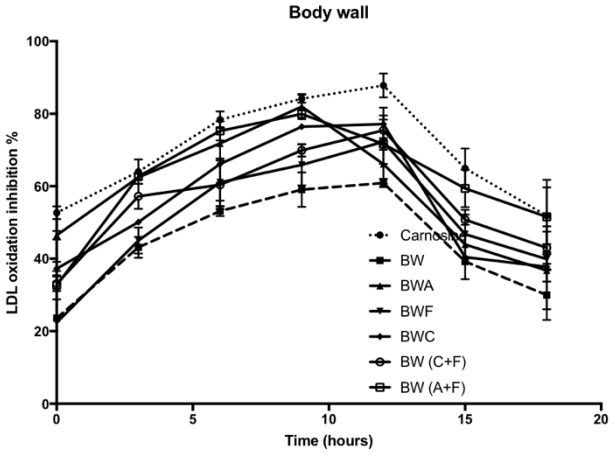
Figure 5.10 shows the inhibition of copper-induced LDL oxidation by carnosine and sea cucumber protein hydrolysates over an 18h of incubation at 37 ºC. Inhibition of LDL cholesterol oxidation of carnosine ranged from ~54% to ~91%. The inhibitory activity of protein hydrolysates prepared from body wall ranged from ~21% to ~80%, whereas inhibition of hydrolysate prepared form the flower was ~23 -78%. The highest inhibition of protein hydrolysates prepared from internal organs observed after 18h incubation was 74%, while the lowest was ~22% for its untreated counterparts. Carnosine exhibited the highest efficacy in inhibiting cupric ion-mediated LDL oxidation (87%), while untreated counterparts of sea cucumber samples showed the lowest inhibitory activity in all three groups (BW, FL, IN). Protein hydrolysates from each group showed stronger inhibitory activity against LDL oxidation compared to the untreated samples. This could be due to the chelating ability and free radical scavenging activity of peptides present in the protein hydrolysates (Ambigaipalan and Shahidi, 2017). Kittiphattanabawon et al. (2013) reported that gelatin hydrolysates prepared from blacktip shark gelatin inhibited human LDL oxidation by 8 - 39%. Amino acid composition plays a vital role in preventing LDL

oxidation. The availability of amino acid residues including tyrosine, histidine, phenylalanine, methionine, glycine, proline or leucine may enhance the chelating and radical scavenging activities of peptides. Park, Jung, Nam, Shahidi and Kim (2001) further explained that peptides containing these amino acids render stronger antioxidant activity against lipid peroxidation than the individual amino acids. A recent study about the influence of peptide charge on the inhibition of LDL oxidation revealed that positively charged peptide fractions possess a significantly higher inhibitory activity than negatively charged fractions (Pan et al., 2019). The effectiveness of amino acid charge on inhibition of LDL oxidation was observed by Wang et al. (2016). These authors suggested that peptides with negatively charged amino acids could prolong the lag time of LDL oxidation. However, in contrast to these findings, it has been suggested that the negatively charged amino acids could also chelate metal ions and may be involved in terminating the free radical chain reaction, as well as preventing the propagation of LDL oxidation (Nwachukwu and Aluko, 2019).

According to the current study, no significant difference (p>0.05) existed among the sea cucumber protein hydrolysates after 12h of incubation. However, the highest inhibitory activities were observed in the protein hydrolysates treated with a combination of enzymes (Alcalase+Flavourzyme and Corolase+Flavourzyme). These results lend support to our findings of the metal chelating ability of protein hydrolysates as described ins section 5.7. Similar inhibitory activity against LDL oxidation was reported for date seed protein hydrolysates (Ambigaipalan and Shahidi 2015). The authors noted that lipophilic antioxidants may extend the LDL oxidation lag phase induced by metal ions. However, the exact mechanism of LDL oxidation inhibition by protein hydrolysates is not yet fully

understood. To date, this is the first study performed to evaluate the potential inhibitory activity of LDL oxidation for sea cucumber protein hydrolysates.

[a]



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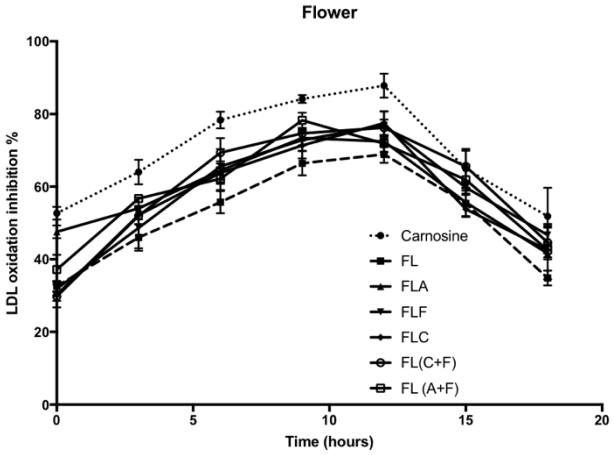


[c]

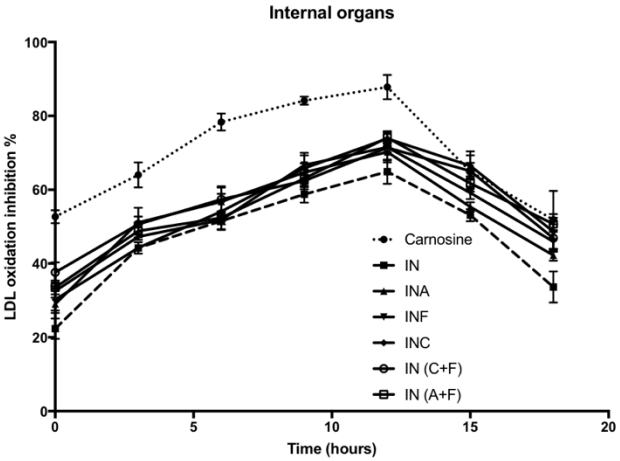


Figure 5.10 Inhibition against human LDL cholesterol oxidation by sea cucumber protein hydrolysates prepared from a) BW, body wall b)FL, flower c) IN, internal organs; A, Alcalase; F, Flavourzyme; C, Corolase; A+F, combination of Alcalase and Flavourzyme; C+F- combination of Corolase and Flavourzyme

5.12 Angiotensin I converting enzyme (ACE) inhibitory activity

ACE inhibitory activity determination was conducted for the samples that exhibited the highest antioxidant activity following enzyme treatment. Thus, samples digested with a combination of Alcalase and Flavourzyme were selected to investigate their antihypertensive activity. Different hydrolysate concentrations 10, 5, and 1 mg/mL were investigated (Figure 5.11). No significant difference (p>0.05) was observed in the ACE inhibitory activities of protein hydrolysates at 10 mg/mL. Hydrolysates prepared using the flower part exhibited a significantly higher (p >0.05) ACE inhibitory activity than body wall and internal organ hydrolysates at 5 and 1 mg/mL. Among all protein hydrolysates, the lowest IC50 value (1.06 mg/mL) was observed for flower protein hydrolysates while internal organ protein hydrolysates showed the highest IC50 value, 1.81 mg/mL. The IC50 value for body wall hydrolysates was 1.66 mg/mL. The observed IC50 values were much higher than that of captopril, a synthetic ACE inhibitor drug with an IC50 of 0.00000478 mg/mL (Lee, Hong, Jeon, Kim and Byun, 2009). Compared to synthetic inhibitors, the reported ACE inhibitory activities of natural peptides are relatively low (Li et al., 2018). However, due to the side effects associated with synthetic drugs, potent natural compounds have received considerable attention for preventing cardiovascular diseases (Lee and Hur, 2017).

Among the various marine sources, sea cucumber protein hydrolysates have gained interest as potential hypertension treatment agents (Ghanbari et al., 2015). Studies on protein hydrolysates from sea cucumber *Actinopyga lecanora* (Ghanbari et al., 2015; Vishkaei et al., 2016), *Acaudina molpadioidea* (Li et al., 2018), *Stichopus horrens*

(Forghani et al., 2016) provide some of the most recent information on potent ACE inhibitory active peptides. The observed IC50 values for protein hydrolysates prepared from sea cucumber (*Cucumaria frondosa*) in the present study were lower than those prepared from sea cucumber *Stichopus horrens* (Forghani et al., 2012). According to Forghani et al. (2012), IC50 value of 2.24 mg/mL for Flavourzyme hydrolysates, 2.28 mg/mL for trypsin hydrolysates, 2.48 mg/mL for papain hydrolysates, and 6.38 mg/ mL for Proteomax hydrolysates were identified in *Stichopus horrens*. In contrast, Alcalase hydrolysates from the same species showed comparatively lower IC50 value (0.48 mg/mL) than present values. This indicates IC50 value of ACE inhibitory activity of sea cucumber protein hydrolysates may depend on the protease type. Therefore, a comparison of the current data with previous studies is rather difficult due to discrepancies in hydrolysis conditions, type of protease used and other related variables. In addition, most studies were conducted using the whole sea cucumber samples, instead of different body parts of the animal. However, the present results are similar with IC50 values obtained from salmon skin collagen hydrolysate (1.165 mg/mL) (Gu, Li, Liu, Yi, and Cai, 2011), cuttlefish hydrolysates (1.58 mg/mL) (Balti, Nedjar-Arroume, Adjé, Guillochon and Nasri, 2010) and goby fish protein hydrolysates (1.36 -3.33mg/mL) (Nasri et al., 2013).

Furthermore, it is noteworthy that low-molecular-weight peptide fractions possess greater ACE inhibitory activity compared to high-molecular-weight peptides and protein hydrolysate mixtures (Ghanbari et al., 2015; Vishkaei et al., 2016). Recent studies have focused on identifying specific peptides responsible for ACE inhibitory activity. The reported IC50 values were comparatively lower than those of high-molecular-weight peptides and protein hydrolysate mixtures (Ambigaipalan and Shahidi, 2017; Li et al.,

2018). Potent peptide inhibitors of ACE have distinct structural features, including hydrophobic amino acid residues at the C-terminal and aliphatic amino acid residues at the N-terminal. In general, it was suggested that ACE inhibitory peptides have short sequences ranging in length from 2 to 12 amino acids (Udenigwe and Aluko, 2012; Ambigaipalan et al., 2015). Thus, further studies are needed to identify the specific peptides responsible for the ACE inhibitory properties of protein hydrolysates prepared from *C .frondosa*.

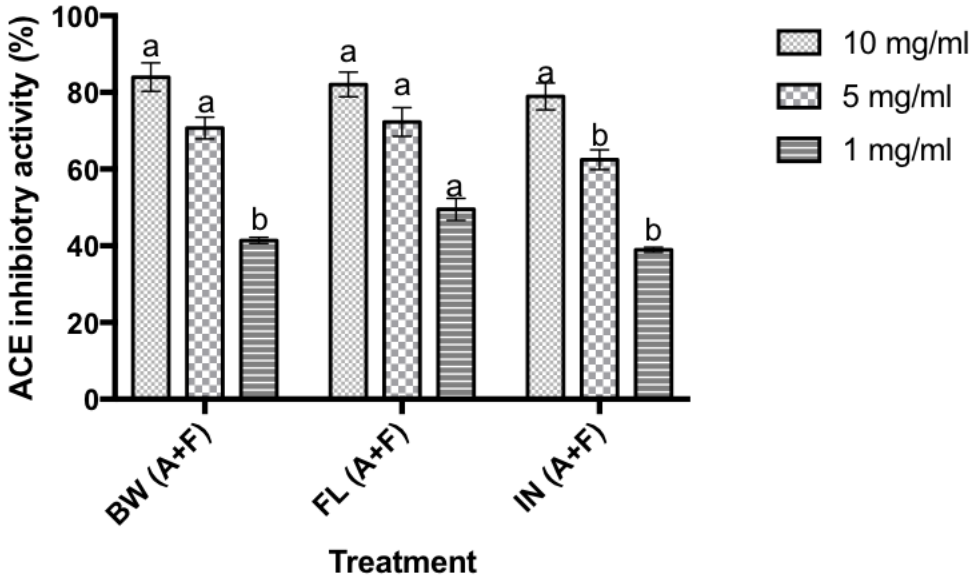


Figure 5.11 ACE inhibitory activity (%) by sea cucumber *C. frondosa* protein hydrolysates (BW, body wall; FL, flower, IN, internal organs using A+F, combination of Alcalase and Flavourzyme). Different letters for the same concentration of all hydrolysates indicate significant difference at $p < 0.05$.

5.13 Color measurement of freeze-dried hydrolysates

Color is one of the most important intrinsic factors that can influence the consumer acceptability of a food product (Wasswa et al., 2007). Protein hydrolysates of sea cucumber samples were analysed for whiteness (L), redness (a) and yellowness (b). L values prepared from body wall were significantly different from their untreated counterpart(Table 5.5). This trend was observed in the other two samples as the highest L values were obtained for untreated flower and internal organs. The lowest L values and highest b values were shown in hydrolysates prepared using Flavourzyme. During hydrolysis, all sea cucumber samples developed a brownish-yellow color which was distinct from their untreated counterparts. Similar findings were reported for protein hydrolysates of round scad (Thiansilakul et al., 2007), grass carp (Wasswa et al., 2007), and sliver carp (Dong et al., 2008). This may be due to the oxidation of myoglobin and melanin pigments of the raw material (Thiansilakul et al., 2007). In addition, as the enzymes such as Flavourzyme have a dark color this may also contribute to the color of the hydrolysis product. Moreover, it has been suggested that the formation of dark color during hydrolysis is associated with the Millard browning process (Dong et al., 2008). Thus, change in color of sea cucumber protein hydrolysates is directly influenced by the muscle pigment oxidation and other factors already noted.

Table 5.4 The color of sea cucumber *C. frondosa* protein hydrolysates

Sample	**L**	**a**	**b**
Body wall			
BW	57.92 ± 0.35^{a}	2.79 ± 0.55^{b}	17.05 ± 0.19^{a}
BWA	55.60 ± 0.72^{b}	3.86 ± 0.58^{a}	18.03 ± 0.88^{a}
BWF	50.59 ± 0.22^{d}	2.70 ± 0.09^{b}	17.32 ± 0.87^{a}
BWC	54.33 ± 0.69^{b}	2.67 ± 0.44^{b}	17.74 ± 0.57^{a}
BW (C+F)	52.62 ± 0.05^{c}	2.91 ± 0.24^{b}	18.14 ± 0.96^{a}
BW (A+F)	56.68 ± 0.47^{a}	3.89 ± 0.46^{a}	18.13 ± 0.78^{a}
Flower			
FL	43.66 ± 0.30^{a}	2.92 ± 0.11^{b}	17.06 ± 0.05^{c}
FLA	42.88 ± 0.15^{b}	3.92 ± 0.32^{a}	18.11 ± 0.62^{b}
FLF	41.26 ± 0.40^{b}	3.25 ± 0.15^{b}	18.91 ± 0.11^{a}
FLC	42.11 ± 0.04^{b}	3.87 ± 0.10^{a}	17.03 ± 0.10^{c}
FL (C+F)	42.46 ± 0.26^{b}	2.34 ± 0.04^{c}	18.99 ± 0.07^{b}
FL (A+F)	41.97 ± 0.28^{b}	3.31 ± 0.33^{b}	18.00 ± 0.29^{a}
Internal organs			
IN	41.89 ± 0.78^{a}	2.92 ± 0.05^{b}	17.01 ± 0.21^{b}
INA	43.25 ± 0.35^{a}	2.14 ± 0.56^{c}	18.18 ± 1.21^{a}
INF	43.13 ± 0.39^{a}	3.35 ± 0.26^{a}	18.05 ± 0.11^{a}
INC	42.34 ± 0.32^{a}	3.65 ± 0.57^{a}	17.58 ± 1.23^{a}
IN (C+F)	43.00 ± 0.33^{a}	3.21 ± 0.70^{a}	18.80 ± 0.89^{a}
IN (A+F)	43.49 ± 0.67^{a}	3.26 ± 0.45^{a}	17.11 ± 0.46^{a}

L, whiteness; a, redness; b, yellowness; BW, body wall; FL, flower; IN, internal organs; A, Alcalase; F, Flavourzyme; C, Corolase; C+F, combination of Corolase and Flavourzyme; A+F, combination of Alcalase and Flavourzyme.
Data are expressed as means ± SD from triplicate determinations.
Different superscripts in each column for the same color parameter of protein hydrolysates prepared from each body part indicate significant difference at $p < 0.05$.

5.14 Solubility of protein hydrolysates

Solubility is one of the key parameters influencing other physicochemical and functional properties of protein hydrolysates (Kristinsson and Rasco, 2000; Ambigaipalan and Shahidi, 2015). The solubility of freeze-dried sea cucumber protein hydrolysates is presented in Figures 5.13 a), b) and c) for body wall, flower, and internal organs, respectively. Figure 5.12 shows the solubility of protein isolates. The solubility of protein isolates showed a typical U-shaped curve, whereas protein hydrolysates exhibited >75% solubility over the pH range of 2-12. There was no significant difference between the enzyme treatments of each sample. However, among all treatments, maximum solubility was achieved in the samples treated with Alcalase and Flavourzyme (body wall 94.34%; flower 90.41%; internal organs 91.43%). In general, solubility improves when pH shifts toward basic conditions. Similar solubility profiles were observed in protein hydrolysates prepared from Pacific whiting muscle (Pacheco-Aguilar, Mazorra-Manzano, and Ramírez-Suárez, 2008), echinoderm by-products (Mamelona et al., 2010b), skipjack roe (Intarasirisawat et al., 2012), cricket (Hall, Jones, O'Haire and Liceaga, 2017) and starry triggerfish muscle (Sripokar, Benjakul and Klomklao, 2019). Change of net charge of the amino acid residues due to alteration of pH and generation of low-molecular-weight peptides during hydrolysis are identified as major factors promoting the solubility of protein hydrolysates over a wide range of pH (Ambigaipalan and Shahidi, 2015; Sripokar, Benjakul and Klomklao, 2019). The enzymatic hydrolysis changes the hydrophobicity of the protein hydrolysates by affecting the balance of hydrophilic and hydrophobic groups of the peptides as well as releasing polar and ionizable groups (Intarasirisawat et al., 2012).

The findings of the present study suggest that protein hydrolysates produced from sea cucumber exhibit better solubility over a wide range of pH compared to their unhydrolyzed counterparts. These results demonstrate the potential of using sea cucumber protein hydrolysates in a wide variety of food systems including emulsions, gels, and foams.

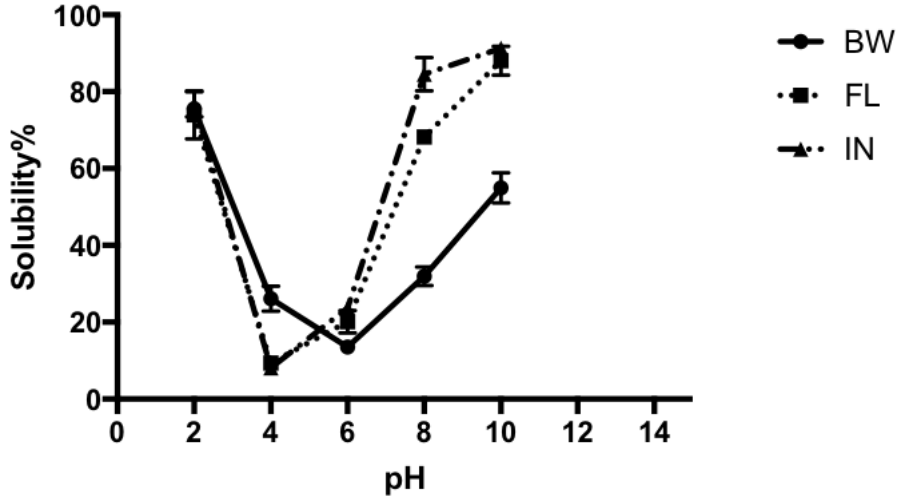
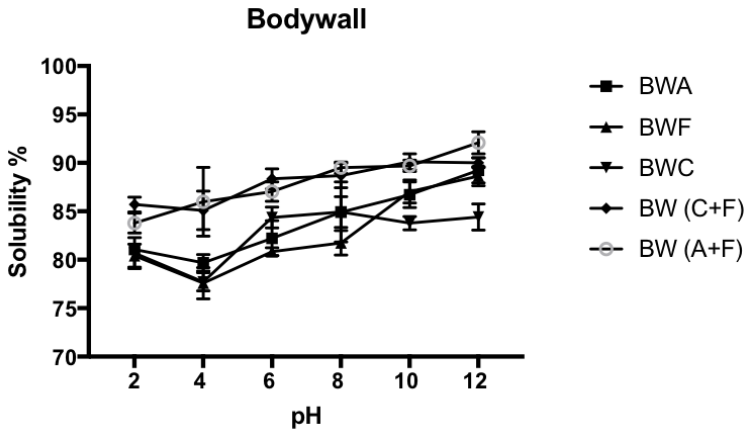
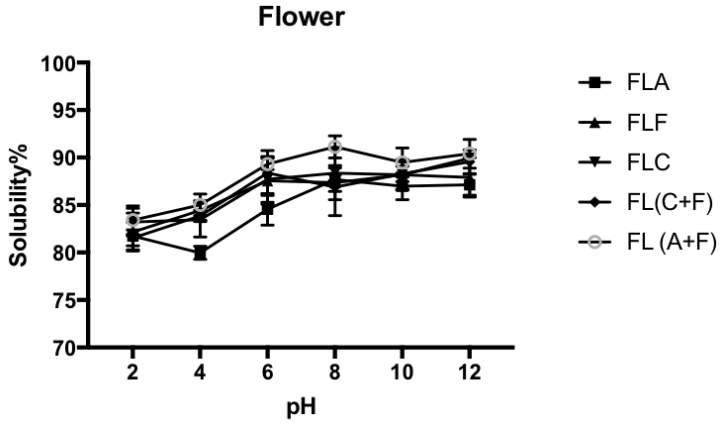


Figure 5.12 Solubility of untreated sea cucumber *C. frondosa* proteins at various pH values BW, body wall; FL, flower; and IN, internal organs

a)



b)



c)

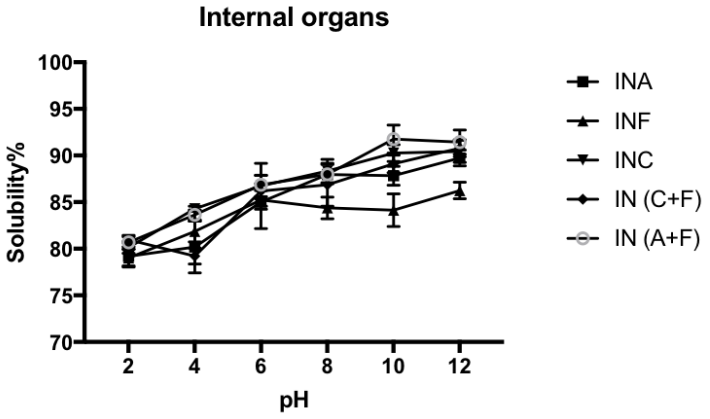


Figure 5.13 Solubility of sea cucumber *C. frondosa* protein hydrolysates at various pH values a) BW, body wall; b) FL, flower; c) IN, internal organs A, Alcalase; F, Flavourzyme; C, Corolase; C+F, combination of Corolase and Flavourzyme; A+F, combination of Alcalase and Flavourzyme.

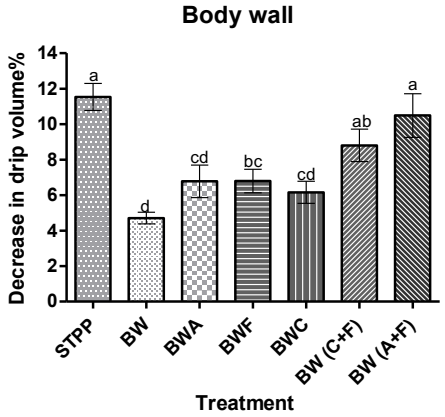
5.15 Determination of water holding capacity (WHC) of protein hydrolysates using a meat model system

Water holding capacity is a key attribute for the textural properties of food products (Wouters et al., 2016). All sea cucumber protein hydrolysates enhanced the WHC of the meat compared with their untreated counterparts (Figure 5.14). Improved WHC correlates with the cooking yield of the meat (Cumby et al., 2008). With respect to the cooking yield, most effective samples were sea cucumber body parts treated with a combination of Alcalase and Flavourzyme and the least effective was unhydrolyzed samples of sea cucumber body parts. These findings are in accordance with the water holding capacity of capelin hydrolysates (Shahidi et al., 1995), rapeseed protein hydrolysates (Cumby et al., 2008) and date seed protein hydrolysates (Ambigaipalan and Shahidi, 2015).

According to Cumby et al. (2008), water holding capacity of food protein hydrolysates depends on the type of protease used for enzymatic hydrolysis, proteolysis condition and availability of low-molecular-weight peptides. In particular, low molecular weight peptide fragments promote the WHC of the protein hydrolysates by decreasing the dripping volume. This may be associated with the greater hydrophilic nature of the low-molecular-weight peptides compared to larger size peptides or unhydrolyzed proteins (Ambigaipalan and Shahidi, 2015). In addition, the presence of polar amino acid residues also contributes to the improved WHC of protein hydrolysates. Cumby et al. (2008) suggested that the use of Flavourzyme promotes the cooking yield of the meat products compared to Alcalase and Colorase by preserving the amino acid residues of the protein hydrolysates. Based on our findings reported here, sea cucumber protein hydrolysates could

be utilized as a natural alternative to sodium tripolyphosphate (STPP) to enhance the cooking yield of meat products, as shown in Figure 5.14.

a)



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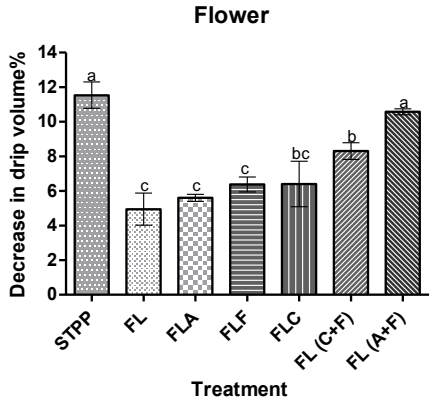

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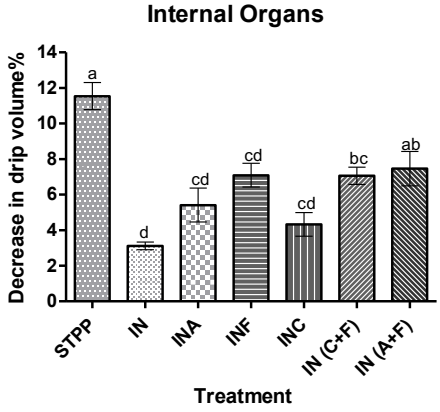

Figure 5.14 Decrease in drip volume of sea cucumber *C. frondosa* protein hydrolysates a) BW, body wall; b) FL, flower; and c) IN, internal organs; A, Alcalase; F, Flavourzyme; C, Corolase; C+F, combination of Corolase and Flavourzyme; and A+F, combination of Alcalase and Flavourzyme. Different letters for the same concentration of all hydrolysates indicate significant difference at $p < 0.05$.

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5.16 Amino acid composition of protein hydrolysates

Consistent with chapter 4…

Amino acid composition plays a vital role in bioactivities and physiochemical properties of the protein hydrolysates. Protein hydrolysates digested with Alcalase and Flavourzyme were further analysed for their total amino acid profiles. As shown in Table 5.6, glutamic acid was dominant in all three samples, followed by glycine in both body wall and flower and arginine in internal organ sample. These findings are consistent with the published amino acid composition for *Cucumaria frondosa* by Zhong et al. (2007), Mamelona et al. (2010a), and Yan et al. (2016). The non-essential amino acid composition (NEAA) of the present study also lend support to the previous findings on different sea cucumber species, including *Parastichopus tremulus* and *Holothuria forskali* (García et al., 2019) and *Holothuria arguinensis* (Roggatz et al., 2016).

Leucine was the most abundant essential amino acid (EAA) found in body wall and flower samples, whereas the essential amino acid with the highest concentration in the internal organ sample was lysine (Table 5.6). Tryptophan, histidine and methionine showed a relatively low amount in all three samples. These results are consistent with those for protein isolates reported in chapter 4. Similar findings were reported in protein hydrolysates produced using Alcalase and trypsin from whole sea cucumber (Zhang et al., 2020). Shahidi et al. (1995) stated that sensitive amino acids, including methionine and tryptophan, may be affected during the analysis process.

However, in terms of nutritional value, all three samples were rich in essential amino acids (30- 40%). The highest ratio EAA/NEAA was shown in internal organ hydrolysates (0.66), followed by flower (0.48) and body wall (0.47). These calculated ratios

well coincide with the findings of Zhong et al. (2007), which demonstrate that the ratio was higher in *Cucumaria frondosa* with internal organs (0.59) compared to the samples devoid of internal organs (0.45, 0.47). Moreover, a similar ratio was reported for sea cucumber guts of *Stichopus japonicus* (0.65) (Du et al., 2019). The ratio EAA /NEAA is considered as an indicator for protein quality of different dietary protein sources (Shahidi, 1994).

As indicated in Table 5.7, amino acid profiles of sea cucumber protein hydrolysates fulfill the recommendations of the World Health Organization /Food and Agriculture Organization (WHO/FAO) 2007 for the amount of most of essential amino acids in food products. The results demonstrate that sea cucumber protein hydrolysates can be used as a source of balanced dietary proteins. All three samples show similar values in terms of their contents of total hydrophobic amino acids and total aromatic amino acids. Hydrophobic amino acids contribute to the antioxidant and ACE inhibitory activities of the protein hydrolysates (Udenigwe et al., 2016; Ambigaipalan and Shahidi, 2017). Zhang et al. (2020) identified tyrosine, methionine, histidine, cysteine and tryptophan as key constituent antioxidative amino acids present in sea cucumber protein hydrolysates. For example, tyrosine residue could serve as a potent hydrogen donor and histidine can be attributed to the metal chelation and lipid radical-trapping ability of the imidazole ring (Thiansilakul et al., 2007).

In addition, findings of Yan et al. (2016) indicated that amino acid residues, including aspartic and glutamic acids, were also responsible for the antioxidant activities displayed by sea cucumber gut *Stichopus japonicus* protein hydrolysates. Negatively charged aspartic and glutamic acids possess excess electrons that can easily be donated and act as free radical scavengers (Nwachukwu and Aluko, 2019). Furthermore, most of the

abundant amino acids in sea cucumber protein hydrolysates are crucial in important physiological functions. For example, glutamine is considered as conditionally essential in catabolic states (Mamelona et al., 2010a); and leucine, isoleucine and valine are important in regulating blood sugar levels and enhancing physical and mental well-being by affecting fatigue conditions (Zhang et al., 2020). Moreover, glycine and low ratios of lysine to arginine have a significant impact on the reduction of serum cholesterol profiles that are directly associated with cardiovascular conditions (Pérez-Vega, Olivera-Castillo, Gómez-Ruiz and Hernández-Ledesma, 2013). The ratio of lysine to arginine for body wall, flower and internal organs were 0.49, 0.52 and 0.78, respectively. These findings imply that amino acid composition could be one of the determinant factors for most of the biological activities exerted by protein hydrolysates of *Cucumaria frondosa* prepared from the sequential addition of Alcalase and Flavourzyme.

Table 5.5 Amino acid composition of protein hydrolysates (Alcalase+Flavourzyme) from different body parts of the North Atlantic sea cucumber (Mole %)

Name	Hydrolysates		
	Body wall	Flower	Internal organs
Essential AA(EAA)			
Histidine (His)-H	1.56	1.70	2.06
Isoleucine (Ile)-I	3.59	3.58	4.27
Leucine (Leu)-L	5.37	5.38	6.45
Lysine (Lys)-K	4.77	5.13	7.74
Methionine (Met)-M	1.85	1.71	2.36
Phenylalanine (Phe)-F	3.91	3.95	4.37
Threonine (Thr)-T	5.18	5.30	5.84
Tryptophan (Trp)-W	1.06	1.10	1.49
Valine (Val)-V	4.54	4.71	5.35
Total EAA	31.84	32.56	39.93
Non EAA(NEEA)			
Alanine (Ala)-A	5.88	5.72	4.93
Arginine (Arg)-R	9.71	9.90	9.90
Aspartic acid +Asparagine (Asp+Asn)-D+N	9.53	9.56	9.16
Cystine (Cys)-C	1.33	1.55	1.79
Glutamic acid +Glutamine (Glu+Gln)-E+Q	14.61	14.50	14.06
Glycine (Gly)-G	11.14	10.56	5.82
Proline (Pro)-P	6.18	5.90	4.27
Serine (Ser)-S	6.09	5.98	5.60
Tyrosine (Tyr)-Y	3.68	3.77	4.53
Total NEAA	68.16	67.44	60.07
EAA/ NEAA	0.47	0.48	0.66
Total polar AA	56.47	57.39	60.68
Total hydrophobic AA	43.53	42.61	39.32
Total Aromatic AA	7.59	7.72	8.91
Branched chain AA	13.50	13.67	16.07

Table 5.6 Comparison of essential amino acid composition of protein hydrolysates from different body parts of the North Atlantic sea cucumber and World Health Organization /Food and Agriculture Organization (WHO/FAO) recommendations (g/100 g)

Amino Acid	WHO/FAO	Hydrolysates		
		Body wall	Flower	Internal organs
Histidine (His)	1.60	0.75	0.87	0.79
Threonine (Thr)	0.90	2.50	2.73	2.22
Valine (Val)	1.30	2.19	2.42	2.04
Methionine (Met)	1.70	0.89	0.88	0.90
Isoleucine (Ile)	1.30	1.73	1.85	1.63
Leucine (Leu)	1.90	2.59	2.77	2.46
Phenylalanine+ Tyrosine (Phe+Tyr)	1.90	5.85	6.40	5.434
Lysine (Lys)	1.60	2.30	2.64	2.95
Tryptophan (Trp)	0.50	0.51	0.57	0.57

5.17 Molecular weight analysis by liquid chromatography- mass spectrometry (LC-MS/MS)

Protein hydrolysates that exhibited the highest antioxidant activity were further analysed to identify their molecular weight distribution. Thus, samples treated with the sequential addition of Alcalase and Flavourzyme were selected from all three body parts of the sea cucumber. LC-MS/MS analysis resulted in the identification of over 400 peptides from each sample. A total of 419 peptides was identified in body wall protein hydrolysates, whereas 538 and 513 peptides were identified from the molecular weight profiles of flower and internal organ protein hydrolysates, respectively. As shown in Table 5.8, among the identified peptides, a large proportion belonged to the molecular weight range of 0.5-1.0 kDa. This accounts for over 60% of the identified peptides from body wall protein

hydrolysate, around 70% of those from flower protein hydrolysates and more than 80% of those from internal organ hydrolysates.

Moreover, peptides less than 0.5 kDa from the identified peptide profiles were relatively lower than those in the range of 0.5-1.0 kDa. Peptides belonging to the smallest molecular weight range (less than 0.5 kDa) from identified peptides made up for less than 30% of total peptides (27. 45% -body wall, 24. 91%- flower and 14.92% -internal organs). It has been suggested that low-molecular-weight peptides are generally associated with the antioxidant potential of protein hydrolysates compared to larger peptides (Shahidi and Zhong 2008; Nwachukwu and Aluko, 2019).

Recent studies on sea cucumber protein hydrolysates have also demonstrated that low- molecular-weight peptides possess better bioactivities than larger peptides. Enzymatic hydrolysis of body wall of *Stichopus japonicus* (Zhou et al., 2012) and *Isostichopus badionotus* (Pérez-Vega et al., 2013), revealed that low-molecular-weight (less than 1.0 kDa) peptides exert antioxidant activities. In addition, enzymatic hydrolysis of body walls of *Isostichopus badionotus* (Pérez-Vega et al., 2013), *Actinopyga lecanora* (Li et al., 2018), and gutted sea cucumbers *Holothuria atra*, *Holothuria leucospilota*, *Bohadschia marmorata* (Dewi et al., 2020) identified peptides less than 3.0 kDa with ACE inhibitory activity. However, in contrast to these findings, Zhang et al. (2017) revealed that larger molecular weight peptides (30 kDa) from sea cucumber *Apostichopus japonicus* eggs also possessed strong antioxidant properties. Similar observations were reported for antioxidative peptides produced using trypsin and Alcalase from Atlantic sea cucumber *Cucumaria frondosa* (Zhang et al., 2020). It was indicated that no particular relationship existed between the antioxidant activity and low- molecular-weight peptides. Overall, these

studies concluded that the molecular weight of peptides is not always the determinant factor for the bioactivities of protein hydrolysates. Factors including chain length, amino acid type, amino acid sequence, specific position or location of certain amino acid residue in the peptide chain, the spatial conformation of peptides may contribute to their bioactive properties including antioxidant and ACE inhibitory activities (Nwachukwu and Aluko, 2019). Therefore, a combination of these factors may be attributed to the difference of bioactivities exerted by sea cucumber protein hydrolysates prepared from body wall, flower and internal organs. The recent trend for discovering bioactive peptides includes bioinformatics approach which is solely based on computer simulation (*in silico*) (Udenigwe, 2014). Thus, the identified peptides (Appendix I) were further analysed using *in silico* tools.

Table 5.7 Molecular weight range of the identified peptides from hydrolysates prepared using mixture of Alcalase and Flavourzyme

Sample	1.5-1.0 kDa	1.0-0.5 kDa	< 0.5 kDa	Total
Body wall	22 (5.25%)	282 (67.30%)	115 (27.45%)	419
Flower	25 (4.65%)	379 (70.45%)	134 (24.91%)	538
Internal organs	18 (3.51%)	420 (81.87%)	75 (14.62%)	513

CHAPTER 6

IN SILICO ANALYSIS OF BIOACTIVE PEPTIDES PRODUCED FROM SEA CUCUMBER PROTEIN HYDROLYSATES – A BIOINFORMATICS APPROACH

6.1 Introduction

Identification of bioactive peptides has evolved over the years from tedious time-consuming conventional processing steps of purification and characterization process into the use of bioinformatic tools to discover the potential precursors of bioactive peptides (Udenigwe, 2014). Use of protein databases and computer-based tools to assess food protein sequences has become an effective and feasible method to discover and identify the bioactive potentials of peptides (Agyei et al., 2016). In general, overall bioinformatics technology is combining biological mass data using computer science, biology, mathematics and statistical analysis methods (Tu, Cheng, Lu and Du, 2018). The *in silico* approach provides tools as databases of protein and peptide sequences, web-based applications for predicting bioactivity and physicochemical properties of proteins and peptides. In addition, identifying structure-functional relationships of peptide as well as programs that enable the theoretical hydrolysis of proteins by calculating quantitative descriptors and recommending the suited enzymes as endopeptidases or exopeptidases to produce potent peptides from native protein sequences are made possible (Kandemir-Cavas, Pérez-Sanchez, Mert-Ozupek and Cavas, 2019). Furthermore, predictive models of gastrointestinal digestion and absorption and safety assessments of biological queries may

assist in managing, curating, and interpreting the information related to functional activities and properties of bioactive peptides (Tu et al., 2018). Thus, predicting the theoretical bioactive profiles and likelihood of specific biological activities minimizes the number of *in vivo* and clinical trials and provides simple but effective means to evaluate and identify the bioactive peptides in a complex protein mixture. Hence, these findings could enhance the application of bioactive peptides in the food, nutrition and pharmaceutical fields. This chapter explores the potent bioactive peptides of sea cucumber protein hydrolysates derived from body wall, flower and internal organs, using *in silico* tools.

6.2 Prediction of bioactive potential of identified peptides from protein hydrolysates samples

Identified peptides (Appendix I) from LC-MS/MS analysis were analysed using "Peptide Ranker (PepRank)" web-based application. PepRank is an *in silico* tool that predicts the probability of a peptide to be bioactive by ranking them within the range between 0 and 1 (Garg, Apostolopoulos, Nurgali and Mishra, 2018). Computational predictions of bioactivity in PepRank generally cover the different classes of bioactive peptides. In addition, it considers the amino acid composition and impact of extracellular status on predictions (Mooney, Haslam, Pollastri and Shields, 2012). Upon submitting the list of identified peptide sequences from each sample, PepRank predicts the probability of the bioactivity of each submitted peptide. The general threshold value is referred as 0.5 and peptides that possess scores above 0.5 are considered to be bioactive (Mooney, Haslam, Holton, Pollastri and Shields, 2013). It is also noteworthy that false positive rates decrease with increasing the threshold values. For example, a false-positive rate of 16% of short

peptides at 0.5 threshold will decrease to 6% by increasing the threshold to 0.8. For long chain peptides this will change from 11% to 6% (Mooney et al., 2012). Furthermore, it may also affect the true-positive rate. Therefore, it is always recommended to select the threshold value carefully based on the purpose of the analysis; i.e., identification of all the true-positives or screening out or reduce the number of false-positives (Mooney et al., 2012; 2013). For our study, we selected 0.9 as the threshold value based on suggestion that the probability of a peptide to be bioactive is significantly high when the predicted score is close to 1 (Garg et al., 2018). As shown in Table 6.1, 10 peptides were over 0.9 threshold score in body wall whereas 8 and 6 peptides were identified from flower and internal organs samples, respectively. Among all the predicted values, flower and internal organs shared the same peptide sequence (GPPGPQWPLDF with 0.96) for the highest predicted bioactivity, whilst body wall had two similar probable bioactive peptide sequences (GPPGPPGPPGPPG and GPPGPQWPLDF), with the score of 0.96. The predicted bioactive peptides were then analysed for their specific bioactive potentials with emphasis on antioxidant and ACE inhibitory activities using BIOPEP data base.

Table 6.1 Bioactive potential of identified peptides according to PEPRANK

Sample	Total identified peptides over 0.9 threshold	PepRank	Peptide sequence
Body wall	10	0.96	GPPGPPGPPGPPG
		0.96	GPPGPQWPLDF
		0.95	PGGPPPPPP
		0.95	PPGGPPPPPPP
		0.94	PPGGPMGPRMP
		0.92	GPGMMGP
		0.91	GPPGPGNAF
		0.90	LPGGPPPPP
		0.90	GPPGASGPLGIAGSM
		0.90	GPPGASGPLGIAGSM
Flower	8	0.96	GPPGPQWPLDF
		0.94	GPPGPRGPTGRMG
		0.94	GPGGPGPGM
		0.93	APDMAFPR
		0.92	GGFPGGPG
		0.92	GPGMMGP
		0.90	GPPGASGPLGIAGSM
		0.90	GPSGPPGP
Internal organs	6	0.96	GPPGPQWPLDF
		0.94	GEPFPKF
		0.93	APDMAFPR
		0.92	PGGPGPGM
		0.92	GPGMMGP
		0.90	DPIFFPS

6.3 *In silico* predictions of potential antioxidative peptides

BIOPEP-UWM data base tool was used to identify the antioxidant potential of highest ranking peptides. Peptide sequences were analysed based on the profiles of potential bioactive peptides reported in the literature and databases as well as the frequency of occurrence of fragments with a given activity (Minkiewicz, Iwaniak and Darewicz, 2019). Identified peptide sequences were submitted to the BIOPEP as a query sequences and selected the "antioxidant activity" as bioactivity of interest. As shown in Table 6.2, out of ten selected peptides, 9 peptides were predicted to be antioxidative peptides. The most common bioactive sequence found in the peptides originated from body wall was GPP (Gly-Pro-Pro). In addition to that, WPL (Trp-Pro-Leu) and MM (Met-Met) were identified as sequences responsible for the antioxidant activity in body wall protein hydrolysates. Previous studies on sea cucumber *Stichopus japonicus* have also confirmed the occurrence of tryptophan and methionine in the identified antioxidant peptide sequences (Zhou et al., 2012). The same authors have reported that a purified sea cucumber peptide was rich in glycine and proline residues with one tryptophan residue in its sequence. They confirmed that GP (glycine-proline) sequence; in particular occurrence of proline in high proportion in the entire peptide enhanced its radical scavenging ability. These findings were well aligned with predictions of the current study.

Tables 6.3 and 6.4 represent the *in silico* predictions of antioxidant potential of amino acid sequences found in flower and internal organs, respectively. According to the BIOPEP-UWM data base, 5 out of 8 peptides from flower and 2 out of 6 peptides from internal organs were identified as potential antioxidative peptides. Interestingly, all the three body

parts of the sea cucumber shared similar bioactive sequences, namely GPP, WPL and MM. In general, amino acid residues including G (glycine), P (proline), W (tryptophan), L (leucine) and M (methionine) are recognised as some of the most established hydrophobic amino acids associated with antioxidant activity (Acquah, Stefano and Udenigwe, 2018). For example, tryptophan is an electron-dense aromatic residue that can contribute to the chelation of prooxidant metal ions. Moreover, the indole side chain of tryptophan can scavenge free radicals by electron donation (Shahidi and Zhong, 2008). Similar findings were reported by Zhang et al. (2020) in Alcalase-produced peptide fractions from Atlantic sea cucumber samples. Furthermore, the authors indicated that antioxidant potential of peptides produced using Alcalase was higher than that of trypsin-produced peptides due to the presence of the above-mentioned amino acid residues in their sequences (Zhang et al., 2020). In addition, the common amino acid sequence found in the three body parts, GPPGPQWPLDF, contained phenylalanine (F). Phenylalanine can also act as a hydrogen donor to scavenge free radicals (Sabeena-Farvin et al., 2016).

As already explained in previous chapters, presence of hydrophobic and aromatic amino acids is considered as a key attribute to the antioxidative property of bioactive peptides (Samaranayaka and Li-Chan, 2011; Chai, Law, Wong, and Kim, 2017). It is also important to consider lipid solubility of peptides as affected by the presence of hydrophobic amino acids that can facilitate their interaction with radical species (Sabeena-Farvin et al., 2016). Therefore, the current findings support the fact that sea cucumber-derived peptides may have the potential to inhibit lipid peroxidation.

The antioxidative peptide (GPEPTGPTGAPQWLR) isolated from sea cucumber *Stichopus japonicus* also confirmed the occurrence of tryptophan and methionine in its

sequence (Zhou, Wang and Jiang, 2012). Furthermore, this purified sea cucumber peptide was rich in glycine and proline residues with one tryptophan residue in its sequence. It was confirmed that GP (glycine-proline) sequence and particularly the occurrence of proline in a high proportion in the entire peptide enhanced its radical scavenging ability, in addition to the direct contribution from the presence of tryptophan (Zhou et al., 2012). Moreover, glycine can function as a potential target site for free radicals. This is further explained by the presence of glycine as a backbone constituent of peptides. It has been reported that peptides having glycine as dominant constituent in its backbone are more flexible, which enhances the exposure of the functional groups to scavenge free radicals (Wu et al., 2018). Another study on autolysis of sea cucumber *Stichopus japonicus* guts suggested that nucleophilic sulfur-containing amino acids like cysteine and methionine in the sequences of peptide exhibits antioxidant properties (Zheng et al., 2012). Notably, exposed methionine residues can act as reactive site to scavenge oxidants through the formation of a sulfoxide structure, thus preventing the propagation of free radical chain reactions (Wu et al., 2018). The specific arrangements of the amino acids within the sequence are crucial for antioxidant activity (Shahidi and Zhong, 2008; Samaranayaka and Li-Chan, 2011). All these findings support the *in silico* predictions of antioxidative peptides produced from body wall, flower and internal organs.

In addition, the synergism of amino acid residues present in the sequence may also contribute to antioxidant properties (Wong et al., 2019). Therefore, the occurrence of potential bioactive sequences inside the identified peptides may enhance opportunities to identify novel antioxidant sources. However, due to the discrepancies of enzyme treatments, variations of species tested, and scarcity of antioxidative peptide information

for sea cucumber reported in the literature, direct comparison of identified sequences is not feasible. Therefore, follow-up studies including wet laboratory synthesis of peptides for the confirmation of bioactivity is needed.

Table 6.2 *In silico* predictions of potential antioxidative peptides from body wall protein hydrolysates

Sample	Peptide Sequence	Name of Peptide	Activity	Bioactive Sequence	Location of Fragmentation
Body wall	GPPGPPGPPGPPG	Antioxidative peptide	antioxidative	GPP	[1-3],[4-6],[7-9],[10-12]
	GPPGPQWPLDF	Peptide from buckwheat	antioxidative	WPL	[7-9]
		Antioxidative peptide	antioxidative	GPP	[1-3]
	PGGPPPPPP	Antioxidative peptide	antioxidative	GPP	[4-6]
	PPGGPPPPPPP	Antioxidative peptide	antioxidative	GPP	[4-6]
	PPGGPMGPRMP	_	_	_	_
	GPGMMGP	Antioxidative peptide	antioxidative	MM	[4-5]
	GPPGPGNAF	Antioxidative peptide	antioxidative	GPP	[1-3]
	LPGGPPPPP	Antioxidative peptide	antioxidative	GPP	[5-7]
	GPPGASGPLGIAGSM	Antioxidative peptide	antioxidative	GPP	[1-3]
	GPSGPPGP	Antioxidative peptide	antioxidative	GPP	[4-6]

Table 6.3 *In silico* predictions of potential antioxidative peptides from flower protein hydrolysates

Sample	Peptide Sequence	Name of Peptide	Activity	Bioactive Sequence	Location of Fragmentation
Flower	GPPGPQWPLDF	peptide from buckwheat	antioxidative	WPL	[7-9]
		Antioxidative peptide	antioxidative	GPP	[1-3]
	GPPGPRGPTGRMG	Antioxidative peptide	antioxidative	GPP	[1-3]
	GPGGPGPGM	–	–	–	–
	APDMAFPR	–	–	–	–
	GGFPGGPG	–	–	–	–
	GPGMMGP	Antioxidative peptide	antioxidative	MM	[5-6]
	GPPGASGPLGIAGSM	Antioxidative peptide	antioxidative	GPP	[1-3]
	GPSGPPGP	Antioxidative peptide	antioxidative	GPP	[4-6]

Table 6.4 *In silico* predictions of potential antioxidative peptides from internal organs hydrolysates

Sample	Peptide Sequence	Name of Peptide	Activity	Bioactive Sequence	Location of Fragmentation
Internal organs	GPPGPQWPLDF	Peptide from buckwheat	antioxidative	WPL	[7-9]
		Antioxidative peptide	antioxidative	GPP	[1-3]
	GEPFPKF	–	–	–	–
	APDMAFPR	–	–	–	–
	PGGPGPGM	–	–	–	–
	GPGMMGP	Antioxidative peptide	antioxidative	MM	[5-6]
	DPIFFPS	–	–	–	–

6.4 *In silico* predictions of potential ACE inhibitory peptides

Computer simulation for the identification of ACE inhibitory activities was conducted using the BIOPEP-UWM data base tool by selecting the "ACE inhibitory activity" as bioactivity of interest. *In silico* approaches use chemometrics and information related to sequence homology for understanding and predicting the bioactivity of amino acid sequences submitted to the data base as "query sequence" (Agyei, Tsopmo and Udenigwe, 2018). According to the findings given in Tables 6.5, 6.6 and 6.7, all selected bioactive peptides in body wall, flower and internal organs possess ACE inhibitory active sequences. It is evident that all active fragments are either dipeptides or tripeptides. Presence of hydrophobic amino acid residues is the crucial structural feature for ACE inhibitory potential (Udenigwe and Aluko, 2012). Hydrophobic amino acids including P (proline), M (methionine), L (leucine), I (isoleucine), F (phenylalanine), A (alanine) were identified in the predicted potent amino acid sequences. In addition, positioning of hydrophobic amino acids or branched chain amino acid residues at the C- terminal of the sequence and N-terminal aliphatic amino acids are some of the major structural features which favors the ACE binding function (Ambigaipalan et al., 2015). These characteristic features were exhibited in predicted sequences of body wall, flower and internal organs. The highest number of potential ACE inhibitory dipeptides, tripeptides (17) were found in sequence GPPGASGPLGIAGSM from body wall and flower.

A number of peptides included in sea cucumber protein hydrolysates have previously been identified as being potent inhibitors of ACE, from various animal and plant sources. For example, according to the database dipeptides GP, PL and tripeptides LPG,

PLG, have previously been identified from Alaskan pollack skin (Table 6.5, 6.6 and 6.7). Furthermore, GPP from wheat gliadin, IA from soy hydrolysate (Tables 6.5 and 6.6), and GEP from *Tricholoma giganteum*are, are some of the other identified potent ACE inhibitory peptides (Table 6.7). Bioactivity of most of these peptides have been verified and confirmed by wet lab synthesis. This demonstrates that sea cucumber protein hydrolysates from body wall, flower and internal organs could also be utilized as potential antihypertensive agents. Furthermore, all the other predicted ACE inhibitory peptides shared similar structural features, including the presence of hydrophobic amino acid residues in the sequence. Ambigaipalan and Shahidi (2017) reported that occurrence of consecutive proline-proline dipeptide in the sequence is markedly effective for antihypertensive activities of shrimp shell discards. Several structure-activity studies have demonstrated the importance of proline in ACE inhibitory activity (O'Keeffe, Norris, Alashi, Aluko and FitzGerald, 2017). The effect of proline towards the ACE inhibitory activity associates with its imidazole ring that exhibits strong interaction with the amino acid residues at the active centers of ACE (Li et al., 2018).

O'Keeffe et al. (2017) stated that more than 50% of the identified ACE inhibitory peptides from gelatin hydrolysates had proline residues in the C-terminal position whereas more than 60% had one proline residue in one of the three C-terminal positions. These findings suggest the importance of locating the proline residue in a favorable position for ACE inhibition (O'Keeffe et al., 2017). These authors further explained the effect of GP (glycine-proline) sequence at the C-terminal of the peptides to exert the ACE inhibitory activity. According to their findings, most of the tri- and tetrapeptides with ACE inhibitory activity were found to a have GP segment at their C-terminal. The identified tripeptides

exhibited significantly higher potency than dipeptides which share the similar structural features (O'Keeffe et al., 2017). Most of the identified peptides in the present study also had this GP sequence at their C-terminal.

A recent investigation on structure-activity relationship of sea cucumber (*Acaudinamolpadioidea*) derived ACE inhibitory peptides indicated that presence of proline residue in the N-terminus significantly enhanced ACE inhibitory activity. These findings are consistent with the observations of *in silico* prediction in the present study. Almost all predicted ACE inhibitory peptides in sea cucumber protein hydrolysates were found to have the proline residue in their sequences. This further confirms the ACE inhibition potency of peptides derived from North Atlantic sea cucumber.

However, it has been suggested that ACE inhibitory property of protein hydrolysates could be considered as a collective effect from various peptide chains rather than from a single bioactive peptide (Ambigaipalan and Shahidi, 2017). In addition, it is always recommended to investigate the potential resistance to gastric enzymes of these identified bioactive peptides, once they are expected to exert a beneficial physiological effect after digestion. Thus, all predicted peptides from sea cucumber protein hydrolysates were subjected to *in silico* simulated gastrointestinal digestion to determine their efficacy upon digestion.

Table 6.5 *In silico* predictions of potential ACE inhibitory active peptides from internal organs hydrolysates

Sample	Peptide Sequence	Name of Peptide	Bioactive Sequence	Location of Fragmentation
Body wall	GPPGPPGPPGPPG	ACE inhibitor from Alaskan pollack skin	GP	[1-2],[4-5],[7-8],[10-11]
		ACE inhibitor	PG	[3-4],[6-7],[9-10],[12-13]
		ACE inhibitor from wheat gliadin	GPP	[1-3],[4-6],[7-9],[10-12]
		ACE inhibitor	PP	[2-3],[5-6],[8-9],[11-12]
	GPPGPQWPLDF	ACE inhibitor from Alaskan pollack skin	GP	[1-2],[4-5]
		ACE inhibitor from Alaskan pollack skin	PL	[8-9]
		ACE inhibitor	PG	[3-4]
		ACE inhibitor from wheat gliadin	GPP	[1-3]
		ACE inhibitor	PP	[2-3]
		ACE inhibitor	PQ	[5-6]
		ACE inhibitor	DF	[10-11]
	PGGPPPPPP	ACE inhibitor from Alaskan pollack skin	GP	[4-5]
		ACE inhibitor	GG	[3-4]
		ACE inhibitor	PG	[2-3]
		ACE inhibitor from wheat gliadin	GPP	[4-6]
		ACE inhibitor	PP	[5-6],[6-7],[7-8],[8-9],[9-10]
		ACE inhibitor	PPP	[5-7],[6-8],[7-9],[8-10]

PPGGPPPPPPP	ACE inhibitor from Alaskan pollack skin	GP	[4-5]
	ACE inhibitor	GG	[3-4]
	ACE inhibitor	PG	[2-3]
	ACE inhibitor from wheat gliadin	GPP	[4-6]
	ACE inhibitor	PP	[1-2],[5-6],[6-7],[7-8],[8-9],[9-10],[10-11]
	ACE inhibitor	PPP	[5-7],[6-8],[7-9],[8-10],[9-11]
PPGGPMGPRMP	ACE inhibitor	PR	[8-9]
	ACE inhibitor from Alaskan pollack skin	GP	[4-5],[7-8]
	ACE inhibitor	MG	[6-7]
	ACE inhibitor	GG	[3-4]
	ACE inhibitor	PG	[2-3]
	ACE inhibitor	PP	[1-2]
	ACE inhibitor	GPM	[4-6]
	ACE inhibitor	MGP	[6-8]
GPGMMGP	ACE inhibitor from Alaskan pollack skin	GP	[1-2],[6-7]
	ACE inhibitor	GM	[3-4]
	ACE inhibitor	MG	[5-6]
	ACE inhibitor	PG	[2-3]
	ACE inhibitor	MM	[4-5]
	ACE inhibitor	MGP	[5-7]

GPPGPGNAF	ACE inhibitor from Alaskan pollack skin	GP	[1-2],[4-5]
	ACE inhibitor	AF	[8-9]
	ACE inhibitor	PG	[3-4],[5-6]
	ACE inhibitor from wheat gliadin	GPP	[1-3]
	ACE inhibitor	PP	[2-3]
LPGGPPPPP	ACE inhibitor from Alaskan pollack skin	LPG	[2-4]
	ACE inhibitor from Alaskan pollack skin	GP	[5-6]
	ACE inhibitor	GG	[4-5]
	ACE inhibitor	PG	[3-4]
	ACE inhibitor from wheat gliadin	GPP	[5-7]
	ACE inhibitor	PP	[6-7],[7-8],[8-9],[9-10]
	ACE inhibitor	PPP	[6-8],[7-9],[8-10]
GPPGASGPLGIAGSM	ACE inhibitor from Alaskan pollack skin	GPL	[7-9]
	ACE inhibitor from Alaskan pollack skin	PLG	[8-10]
	ACE inhibitor from Alaskan pollack skin	GP	[1-2],[7-8]
	ACE inhibitor from Alaskan pollack skin	PL	[8-9]
	ACE inhibitor from soy hydrolysate	IA	[11-12]
	ACE inhibitor	GI	[10-11]
	ACE inhibitor	GA	[4-5]

	ACE inhibitor	AG	[12-13]
	ACE inhibitor	GS	[13-14]
	ACE inhibitor	SG	[6-7]
	ACE inhibitor	LG	[9-10]
	ACE inhibitor	PG	[3-4]
	ACE inhibitor from wheat gliadin	GPP	[1-3]
	ACE inhibitor	PP	[2-3]
	ACE inhibitor	LGI	[9-11]
	ACE inhibitor	SGP	[6-8]
	ACE inhibitor	AGS	[12-14]
GPSGPPGP	ACE inhibitor from Alaskan pollack skin	GP	[1-2],[4-5],[7-8]
	ACE inhibitor	SG	[3-4]
	ACE inhibitor	PG	[6-7]
	ACE inhibitor from wheat gliadin	GPP	[4-6]
	ACE inhibitor	PP	[5-6]
	ACE inhibitor	SGP	[3-5]

Table 6.6 *In silico* predictions of potential ACE inhibitory active peptides from flower protein hydrolysates

Sample	Peptide Sequence	Name of Peptide	Bioactive Sequence	Location of Fragmentation
Flower	GPPGPQWPLDF	ACE inhibitor from Alaskan pollack skin	GP	[1-2],[4-5]
		ACE inhibitor from Alaskan pollack skin	PL	[8-9]
		ACE inhibitor	PG	[3-4]
		ACE inhibitor from wheat gliadin	GPP	[1-3]
		ACE inhibitor	PP	[2-3]
		ACE inhibitor	PQ	[5-6]
		ACE inhibitor	DF	[10-11]
	GPPGPRGPTGRMG	ACE inhibitor	PR	[5-6]
		ACE inhibitor from Alaskan pollack skin	GP	[1-2],[4-5],[7-8]
		ACE inhibitor	GR	[10-11]
		ACE inhibitor	MG	[12-13]
		ACE inhibitor	TG	[9-10]
		ACE inhibitor	PG	[3-4]
		ACE inhibitor from wheat gliadin	GPP	[1-3]
		ACE inhibitor	PT	[8-9]
		ACE inhibitor	PP	[2-3]
		ACE inhibitor	RG	[6-7]
	GPGGPGPGM	ACE inhibitor from Alaskan pollack skin	GP	[1-2],[4-5],[6-7]
		ACE inhibitor	GM	[8-9]

	ACE inhibitor	GG	[3-4]
	ACE inhibitor	PG	[2-3],[5-6],[7-8]
APDMAFPR	ACE inhibitor	FP	[6-7]
	ACE inhibitor	PR	[7-8]
		AFP	[5-7]
	ACE inhibitor	AF	[5-6]
	ACE inhibitor	AP	[1-2]
	ACE inhibitor	DM	[3-4]
GGFPGGPG	ACE inhibitor	FP	[4-5]
	ACE inhibitor from Alaskan pollack skin	GP	[7-8]
	ACE inhibitor	GF	[3-4]
	ACE inhibitor	GG	[2-3],[6-7]
	ACE inhibitor	PG	[5-6],[8-9]
GPGMMGP	ACE inhibitor	GM	[4-5]
	ACE inhibitor	MG	[6-7]
	ACE inhibitor	PG	[3-4]
	ACE inhibitor	MM	[5-6]
	ACE inhibitor	MGP	[6-8]
GPPGASGPLGIAGSM	ACE inhibitor from Alaskan pollack skin	GPL	[7-9]
	ACE inhibitor from Alaskan pollack skin	PLG	[8-10]
	ACE inhibitor from Alaskan pollack skin	GP	[1-2],[7-8]

	ACE inhibitor from Alaskan pollack skin	PL	[8-9]
	ACE inhibitor from soy hydrolysate	IA	[11-12]
	ACE inhibitor	GI	[10-11]
	ACE inhibitor	GA	[4-5]
	ACE inhibitor	AG	[12-13]
	ACE inhibitor	GS	[13-14]
	ACE inhibitor	SG	[6-7]
	ACE inhibitor	LG	[9-10]
	ACE inhibitor	PG	[3-4]
	ACE inhibitor from wheat gliadin	GPP	[1-3]
	ACE inhibitor	PP	[2-3]
	ACE inhibitor	LGI	[9-11]
	ACE inhibitor	SGP	[6-8]
	ACE inhibitor	AGS	[12-14]
GPSGPPGP	ACE inhibitor from Alaskan pollack skin	GP	[1-2],[4-5],[7-8]
	ACE inhibitor	SG	[3-4]
	ACE inhibitor	PG	[6-7]
	ACE inhibitor from wheat gliadin	GPP	[4-6]
	ACE inhibitor	PP	[5-6]
	ACE inhibitor	SGP	[3-5]

Table 6.7 *In silico* predictions of potential ACE inhibitory active peptides from internal organs hydrolysates

Sample	Peptide Sequence	Name of Peptide	Bioactive Sequence	Location of Fragmentation
Internal organs	GPPGPQWPLDF	ACE inhibitor from Alaskan pollack skin	GP	[1-2],[4-5]
		ACE inhibitor from Alaskan pollack skin	PL	[8-9]
		ACE inhibitor	PG	[3-4]
		ACE inhibitor from wheat gliadin	GPP	[1-3]
		ACE inhibitor	PP	[2-3]
		ACE inhibitor	PQ	[5-6]
		ACE inhibitor	DF	[10-11]
	GEPFPKF	ACE inhibitor	FP	[4-5]
		ACE inhibitor from Tricholoma giganteum	GEP	[1-3]
		ACE inhibitor	GE	[1-2]
		ACE inhibitor	KF	[6-7]
		ACE inhibitor	PFP	[3-5]
	APDMAFPR	ACE inhibitor	FP	[7-8]
		ACE inhibitor	PR	[8-9]
			AFP	[6-8]
		ACE inhibitor	AF	[6-7]
		ACE inhibitor	AP	[2-3]
		ACE inhibitor	DM	[4-5]

PGGPGPGM	ACE inhibitor from Alaskan pollack skin	GP	[3-4],[5-6]
	ACE inhibitor	GM	[7-8]
	ACE inhibitor	GG	[2-3]
	ACE inhibitor	PG	[1-2],[4-5],[6-7]
GPGMMGP	ACE inhibitor from Alaskan pollack skin	GP	[2-3],[7-8]
	ACE inhibitor	GM	[4-5]
	ACE inhibitor	MG	[6-7]
	ACE inhibitor	PG	[3-4]
	ACE inhibitor	MM	[5-6]
	ACE inhibitor	MGP	[6-8]
DPIFFPS	ACE inhibitor	FP	[5-6]
	ACE inhibitor	IF	[3-4]

6.5 *In silico* simulated gastrointestinal (GI) digestion of bioactive peptides

The desirable physiological effects of bioactive peptides mainly depend on their ability to pass through the intestinal epithelium and reach their target organs in an intact form (Shahidi and Zhong, 2008; Udenigwe and Aluko, 2012; Onuh and Aluko, 2019). Therefore, most researchers have estimated the bioactive capacity of peptides by following simulated GI digestion. Simulated GI digestion that mimics protein degradation in the stomach and small intestine is considered as a rapid and effective tool to determine the stability of peptides against GI proteases (Nwachukwu and Aluko, 2019). In this regard, *in silico* simulated GI digestion is an effective approach to investigate the bioactive potential of any peptide of interest prior to assessing their bioavailability and bioaccessibility *in vivo*. Thus, selected peptides were subjected to *in silico* proteolysis simulation with representative digestive enzymes; pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) using "BIOPEP Enzyme(s) Action" program (Minkiewicz et al., 2019).

The current study predicted the potential of peptides derived from sea cucumber protein hydrolysates as the precursors of peptides with antioxidative or ACE inhibitory properties. Tables 6.8, 6.9 and 6.10 represent the bioactive potential upon GI digestion of peptides derived from body wall, flower and internal organs, respectively. According to the predictions, none of the peptides showed antioxidative potential after the simulated digestion process. This may be due to the scarcity of information in databases related to the potent antioxidatively active peptides. It is also possible that some peptides may undergo cleavage when subjected to GI tract conditions. However, it has been suggested that

bioinformatics strongly depend on the information included in the database (Udenigwe, 2014).

In contrast to the results found for antioxidant potential, prediction of releasing ACE inhibitory peptides was similar in all three samples (Tables 6.5, 6.6 and 6.7). Out of the 10 peptides identified from body wall, only 3 peptides (GPPGPQWPLDF, GPGMMGP and GPPGPGNAF) were predicted to have ACE inhibitory potential. The results for bioactive peptides from flower and internal organs samples were identical as predicted by the *in silico* method. Three peptides (GPPGPQWPLDF, APDMAFPR and GPGMMGP) from flower and internal organs were predicted to release potent ACE inhibitory fragments from their original sequences. GPPGPQWPLDF and GPGMMGP which were the common sequences for all three samples, has been predicted to release potent ACE inhibitory peptides upon simulated GI digestion. Furthermore, frequency of releasing potent ACE inhibitory peptides from each identified peptide was varied as some peptides have more than one active fragment embedded in their sequences. For example, GPPGPQWPLDF sequence was predicted to release two active ACE inhibitory fragments, namely PL and DF, whereas APDMAFPR has the potential to release PR and AF following simulated GI digestion.

The release of bioactive peptides was predicted after using the "enzyme(s) action" option of BIOPEP data base. Calculated quantitative parameters of proteolysis include theoretical degree of hydrolysis (DH_t), the frequency of the release of fragments with a given activity by selected enzymes (A_E), and the relative frequency of the release of fragments with a given activity by selected enzymes (W) (Table 6.11). The DH_t values for peptides varied from 11.11 to 33.33%. Among them, GPGMMGP showed the highest efficiency (33.33%) on releasing the bioactive enzymes and GPPGPGNAF was the lowest

(11.11%). Descriptors like A_E and W directly analyzed the potential bioactivity of sea cucumber derived peptides. The higher A_E values suggest the possibility of having higher number of peptides with specific activity (Iwaniak, Minkiewicz, Pliszka, Mogut and Darewicz, 2020). The highest A_E value (0.25) was observed in APDMAFPR from flower and internal organs, whereas the lowest (0.10) was GPPGPGNAF from body wall.

The other remaining peptide sequences, namely GPPGPQWPLDF and GPGMMGP, were common for all samples and their calculated A_E values were 0.18 and 0.14, respectively. The highest relative frequency of the release of fragments with a given activity by selected enzymes(W) was 0.33 in APDMAFPR, followed by GPPGPQWPLDF (0.25). Similar W value (0.14) was observed for GPGMMGP and GPPGPGNAF. It was suggested that W values are considered complimentary to AE values (Iwaniak et al., 2020). In addition, W values can be correlated with catalytic specificities and the number of recognition sites in each enzyme (Ji et al., 2019). Most of the bioactive fragments remaining after *in silico* simulated digestion of sea cucumber derived peptides possess either P (proline) or phenylalanine (F) in their dipeptide sequences. The resultant bioactive motifs contain PL (proline-leucine), DF (aspartic acid-phenylalanine), GP(glycine-proline), AF (alanine-phenylalanine) and PR (proline-arginine). Similar findings were reported by Iwaniak et al. (2020) in their *in silico* study of identifying biopeptides from collagen derived from various sources including cow, pig, sheep, chicken, duck, horse, salmon, rainbow trout, goat, rabbit and turkey. Authors reported that all the identified motifs which exhibited ACE inhibitory activity contained proline, phenylalanine, glycine, leucine and arginine. As described earlier, previous studies on ACE inhibitory peptides also revealed that proline residues appear to provide resistance to digestive enzymes. The dipeptide sequences with

a C-terminal proline and hydroxyproline were reported to be more bioavailable compared to other amino acid sequences (O'Keeffe et al., 2017). Specifically, the presence of proline is well documented for its distinct ability for binding to ACE and interestingly, most commercially existing inhibitors bear the proline residue in their sequences (Wu, Aluko and Nakai, 2006). Furthermore, some studies have identified that the presence of hydrophobic amino acids at the penultimate position of C-terminus as being a favourable feature for ACE inhibitory potency (Yao, Agyei and Udenigwe, 2018).

Thus, *in silico* prediction of generating potent ACE inhibitory peptides from sea cucumber samples may contribute to a better understanding of peptide activity before performing *in vitro* and *in vivo* experiments. Nevertheless, these findings can be used as a preliminary information on the stability of the sea cucumber derived peptides as well as to help determining the relationship between amino acid composition of peptides and their GI digestion.

Table 6.8 Remaining bioactive properties after *in silico* simulated gastrointestinal digestion of peptides originated from body wall protein hydrolysates

Sample	Peptide	Results of Enzyme Action	Location of Released Peptides	Active Fragment Sequence	Location	Bioactivity of Identified peptide
Body wall	GPPGPPGPPGPPG	-	-	-	-	-
	GPPGPQWPLDF	GPPGPQW - PL - DF	[1-7], [8-9][10-11]	PL	[8-9]	ACE inhibitor
				DF	[10-11]	ACE inhibitor
	PGGPPPPPP	-	-	-	-	-
	PPGGPPPPPPP	-	-	-	-	-
	PPGGPMGPRMP	PPGGPM - GPR - M - P	[1-6],[7-9],[10-10], [11-11]	-	-	No Antioxidant or ACE inhibitory activity
	GPGMMGP	GPGM - M - GP	[1-4],[5-5],[6-7]	GP	[6-7]	ACE inhibitor
	GPPGPGNAF	GPPGPGN - AF	[1-8],[9-10]	AF	[9-10]	ACE inhibitor
	LPGGPPPPP	L - PGGPPPPP	[1-1],[2-9]	-	-	No Antioxidant or ACE inhibitory activity
	GPPGASGPLGIAGSM	GPPGASGPL - GIAGSM	[1-9],[10-15]	-	-	No Antioxidant or ACE inhibitory activity
	GPSGPPGP	-	-	-	-	-

Table 6.9 Remaining bioactive properties after *in silico* simulated gastrointestinal digestion of peptides originated from flower protein hydrolysates

Sample	Peptide	Results of Enzyme Action	Location of Released Peptides	Active Fragment Sequence	Location	Bioactivity of Identified peptide
Flower	GPPGPQWPLDF	GPPGPQW - PL - DF	[1-7], [8-9][10-11]	PL	[8-9]	ACE inhibitor
				DF	[10-11]	ACE inhibitor
	GPPGPRGPTGRMG	GPPGPR - GPTGR - M - G	[1-6],[7-11], [12-12],[13-13]	_	_	No Antioxidant or ACE inhibitory activity
	GPGGPGPGM	_	_	_	_	_
	APDMAFPR	APDM - AF - PR	[1-4],[5-6],[7-8	PR	[7-8]	ACE inhibitor
				AF	[5-6]	ACE inhibitor
	GGFPGGPG	GGF - PGGPG	[1-3],[4-8]	_	_	No Antioxidant or ACE inhibitory activity
	GPGMMGP	GPGM - M - GP	[1-4],[5-5],[6-7]	GP	[6-7]	ACE inhibitor
	GPPGASGPLGIAGSM	GPPGASGPL - GIAGSM	[1-9],[10-15]	_	_	No Antioxidant or ACE inhibitory activity
	GPSGPPGP	_	_	_	_	_

Table 6.10 Remaining Antioxidant or ACE inhibitory properties after *in silico* simulated gastrointestinal digestion of peptides originated from internal organs protein hydrolysates

Sample	Peptide	Results of Enzyme Action	Location of Released Peptides	Active Fragment Sequence	Location	Bioactivity of Identified peptide
Internal organs	GPPGPQWPLDF	GPPGPQW - PL - DF	[1-7], [8-9][10-11]	PL	[8-9]	ACE inhibitor
				DF	[10-11]	ACE inhibitor
	GEPFPKF	GEPF - PK - F	[1-4],[5-6],[7-7]	_	_	No Antioxidant or ACE inhibitory activity
	APDMAFPR	APDM - AF - PR	[1-4],[5-6],[7-8]	PR	[7-8]	ACE inhibitor
				AF	[5-6]	ACE inhibitor
	PGGPGPGM	_	_	_	_	_
	GPGMMGP	GPGM - M - GP	[1-4],[5-5],[6-7]	GP	[6-7]	ACE inhibitor
	DPIFFPS	DPIF - F - PS	[1-4],[5-5],[6-7]	_	_	No Antioxidant or ACE inhibitory activity

Table 6.11 *In silico* hydrolysis performance and physicochemical characteristics of bioactive peptides remaining after simulated in silico digestion

Peptide	Sample	Active Fragment Sequence	Location	DHt (%)	A_E	W
GPPGPQWPLDF	body wall, flower, internal organs	PL DF	[8-9] [10-11]	20	0.18	0.25
GPGMMGP	body wall, flower internal organs	GP	[6-7]	33.33	0.14	0.14
APDMAFPR	flower, internal organs	AF PR	[5-6] [7-8]	28.57	0.25	0.33
GPPGPGNAF	body wall	AF	[9-10]	11.11	0.10	0.14

6.6 Prediction of toxicity and physicochemical properties of released bioactive peptide fractions after *in silico* digestion

In silico proteolysis does not guarantee that the resultant peptides with bioactivities are always safe for use in food and pharmaceutical products. In addition, there is a possibility of identifying novel peptides. Therefore, toxicity evaluation has been suggested as a mandatory approach prior to further development of identified motifs (Ji et al., 2019). The software ToxinPred was used for predicting the toxicity of peptides as well as their physicochemical properties including hydrophobicity, hydrophilicity, charge, isoelectric point and molecular weight of the released dipeptides. The toxicity of peptides was investigated based on hybrid model of dipeptide composition and motif scanning (Lafarga, O'Connor and Hayes, 2014). According to the model developed using the machine-learning techniques support vector machine (SVM) based on amino acid compositional analysis, residue preferences combined with quantitative matrix-based and motif-based predictions, ToxinPred creates the platform to assess realistic toxicity of peptides (Gupta et al., 2013). The developed method revealed that valine (V), threonine (T), arginine (R), glutamine (E), methionine (M), leucine (L), lysine (K), isoleucine (I), phenylalanine (F) and alanine (A) were abundant in non-toxic peptides, whereas cysteine (C), histidine (H) and asparagine (N) were predominant in toxic peptides (Gupta et al., 2013). Predictions obtained following toxicity analysis have shown that none of the dipeptides (PL, GF, GP, DF, PR) released from sea cucumber-derived peptide simulated digestion had potential toxicity. Thus, these findings indicated that sea cucumber-derived peptides can be used as functional food ingredients or nutraceutical products.

According to the predictions of ToxinPred, molecular weight of the peptides ranged from 172.20 to 280.29 Da. Out of the five identified ACE inhibitory potent dipeptides obtained from sea cucumber protein hydrolysates, three (PL, GP and AF) had an isoelectric point (pI) of 5.88 with 0 net charge (Table 6.12). The remaining peptides, DF and PR, had negative charge (-1) and positive charge (+1) with a pI of 3.88 and 10.11, respectively. Structural properties, including charge, peptide sequence and low molecular size have been identified as influential characteristics for the bioavailability of food-derived peptides (Sun, Acquah, Aluko and Udenigwe, 2020). It has been reported that short peptides produced from gelatin hydrolysates have the potential to enter the blood stream by crossing the intestinal barriers of humans and exert their bioactivity (Iwai et al., 2005). Generally, small peptide sequences are less prone to further degradation and possess high bioavailability (Ryder, Bekhit, McConnell and Carne, 2016). In addition, most of the reported potent ACE inhibitory peptides are di- or tripeptides with the ability of binding to the buried active site of ACE (Ryder et al., 2016). Limited information is available in the literature that discusses correlation between the isoelectric point of peptides and their ACE inhibitory activity. However, a recent study on peptides derived from winged bean seeds showed that peptides in acidic (4.16-5.86 pH) and basic (9.38-10 pH) regions had stronger ACE inhibitory activity compared to peptides with neutral pI (Yea et al., 2014). Moreover, a study of structure-functional activity relationship on ACE inhibitory protein indicated that C-terminus in a dipeptide is more important than the N-terminus. Moreover, C-terminus has a direct influence on steric properties and lipophilicity of dipeptides (Wu et al., 2006).

Hydrophobicity values of identified bioactive dipeptides ranged from -0.05 to 0.43, while hydrophilicity values were in the range of -0.9 to 0.25. PL showed the highest

hydrophobicity value (0.23) and lowest hydrophilicity value (-0.9). Therefore, the varying physicochemical parameters such as hydrophobicity and hydrophilicity indicate the potential of utilizing the predicted short-chain peptides derived from sea cucumber in a wide range of food systems, including water soluble, lipid soluble as well as in emulsions. In general, ACE inhibitory peptides are expected to exert their functionality in both hydrophilic and hydrophobic systems (Ji, Xu, Udenigwe and Agyei, 2020).

Table 6.12 Physicochemical properties and toxicity of bioactive fragments releasing after the simulation *in silico* digestion

Active fragment sequence	Prediction	Hydrophobicity	Hydrophilicity	Charge	pI	Molecular weight (Da)
PL	Non-toxic	0.23	-0.9	0	5.88	228.31
DF	Non-toxic	-0.05	0.25	-1	3.8	280.29
GP	Non-toxic	0.04	0	0	5.88	172.20
AF	Non-toxic	0.43	-1.5	0	5.88	236.28
PR	Non-toxic	-0.92	1.5	1	10.11	271.33

6.7 *In silico* analysis of absorption, distribution, metabolism and excretion (ADME) profile of bioactive peptides derived from sea cucumber hydrolysates

The trend of using virtual screening for identifying the potential bioactive compounds has become an asset in drug discovery process (Geromichalos, 2012). Drug development assessments generally include absorption, distribution, and excretion (ADME) parameters. The web-based tool SwissADME provides robust predictive models and primary information for individual parameters important for drug development process (Daina, Michielin, and Zoete, 2017). The employment of user-friendly computer-based applications is considered as being a major breakthrough in drug discovery, design and development processes (Ji et al., 2019). Evaluation of ADME parameters at the initial stage mitigates the pharmacokinetics (i.e. the fate of a therapeutic compound in the organism) related failures in the clinical phase. In addition, *in silico* ADME prediction method is recognised as a cost-effective alternative to laborious experimental approaches (Geromichalos, 2012; Daina et al., 2017). SwissADME evaluation methods include the analysis of drug-likeness which investigate the probability to be an oral drug (Daina et al., 2017). One of the most common and convenient delivery methods of peptide therapeutics is oral administration (Yap and Gan, 2020).

The selected criteria in the present study focused on the crucial physicochemical parameters used for drug designing, including number of rotatable bonds (ROTB), number of hydrogen bond acceptors, number of hydrogen bond donors and water solubility. Furthermore, polarity of the molecule was evaluated using topological polar surface area (TPSA) technique considering sulfur and phosphorus as polar atoms. TPSA is one of the

key parameters to estimate the ADME properties (Daina et al., 2017). Lipophilicity was analysed based on the partition coefficient between octanol and water (log Po/w) and it has a vital impact on pharmacokinetics in drug discovery process. Bioavailability score and Lipinski filter were evaluated to assess the qualitative chance for sea cucumber derived peptides to become oral drugs with emphasis on bioavailability and structural characteristics. Lipinski filter is developed on the Lipinski (Pfizer's) rule of five which is known as the rule of thumb to determine the drug-likeness of a molecule (Ji et al., 2019). The main criteria of the rule include less than five hydrogen bond donors (the total number of nitrogen-hydrogen and oxygen-hydrogen bonds), no more than 10 hydrogen bond acceptors (all nitrogen or oxygen atoms), molecular mass of less than 500 Da, and an octanol-water partition coefficient (log *P*) greater than or equal to five. In general, orally-active drugs can have only one exemption/violation of these criteria (Lipinski, Lombardo, Dominy and Feeney, 2012). All these parameters were evaluated on par with the standard captopril.

As shown in Table 6.13, four of the five predicted dipeptides from sea cucumber protein hydrolysates were in accordance with all Lipinski criteria. The remaining dipeptide PR was also in agreement with Lipinski filter criteria with one violation (exceed the maximum number of hydrogen bond donors (HBD)). Moreover, it has been suggested that compounds with more than 10 rotatable bonds show poor oral bioavailability whereas TPSA value ranging from 20 to 130Å^2 are suitable for providing high oral bioavailability (Ji et al., 2020). All peptides, except PR, exhibited low TPSA values and high gastrointestinal absorbance ability. Lipophilicity and polarity of compounds are crucial to determine the passive gastrointestinal digestion (Daina and Zoete, 2016). Furthermore,

bioavailability scores of all the identified peptides were similar to that of standard inhibitory drug captopril.

Bioavailability radar, a graphical representation of most of the above-mentioned parameters with some additional properties simplifies the evaluation of drug-likeness of a molecule (Daina et al., 2017). Six physicochemical properties, including lipophilicity, molecular weight/size, polarity, solubility, saturation, and flexibility of the molecule were displayed in the radar (Figure 6.1). The pink area in the radar represents the favorable range for oral bioavailability which is based on the optimal values for each property. For example, lipophilicity which is indicated by the partition coefficient range between n-octanol and water (log Po/w), should range from -0.7 to +5.0. The molecular weight should be within the size of 150-500 Da and optimal range of TPSA should fall into 20- 130 $Å^2$. In addition, solubility which is denoted using the descriptor decimal logarithm of the molar solubility in water should not be higher than 6, whereas the fraction of carbons in the sp^3 hybridization should not exceed 0.25. The optimum flexibility is accountable for having less than 9 rotatable bonds. However, the crucial determinants related to oral bioavailability of the molecule are flexibility and polarity (Ji et al., 2019). In our study, except PR, all other dipeptides fall into the optimal range of each parameter (Figure 6.1). These findings suggest that identified bioactive dipeptides, excluding PR, have excellent conditions for oral bioavailability and better absorption ability in the GI tract. Thus, SwissADME classifiers predictions demonstrate that peptides derived from sea cucumber hydrolysates may possess drug-like properties, similar to standard inhibitory drugs (captoptril) and have the potential to be used in the pharmaceutical industry. However, further experimental validation is needed for confirmation of the predicted properties.

Table 6.13 *In silico* absorption, distribution, metabolism, excretion (ADME), and physicochemical properties of bioactive peptides

Active Fragment Sequence	Physicochemical properties				Lipophilicity		Drug likeliness		Pharmacokinetics
	ROTB	HBA	HBD	ESOL	TPSA (Å²)	C LogP	Bioavailability score	Lipinski filter	GIA
Captopril	4	3	1	-1.14 Very soluble	96.41	0.62	0.56	Yes(0)	High
PL	6	4	3	0.66 Highly soluble	78.43	0.04	0.55	Yes (0)	High
DF	8	6	4	0.83 Highly soluble	129.72	-0.69	0.56	Yes (0)	High
GP	3	4	2	1.25 Highly soluble	83.63	-1.17	0.55	Yes(0)	High
AF	6	4	3	0.39 Highly soluble	92.42	0.05	0.55	Yes(0)	High
PR	9	5	6	1.94 Highly soluble	140.33	-1.59	0.55	Yes(1)	Low

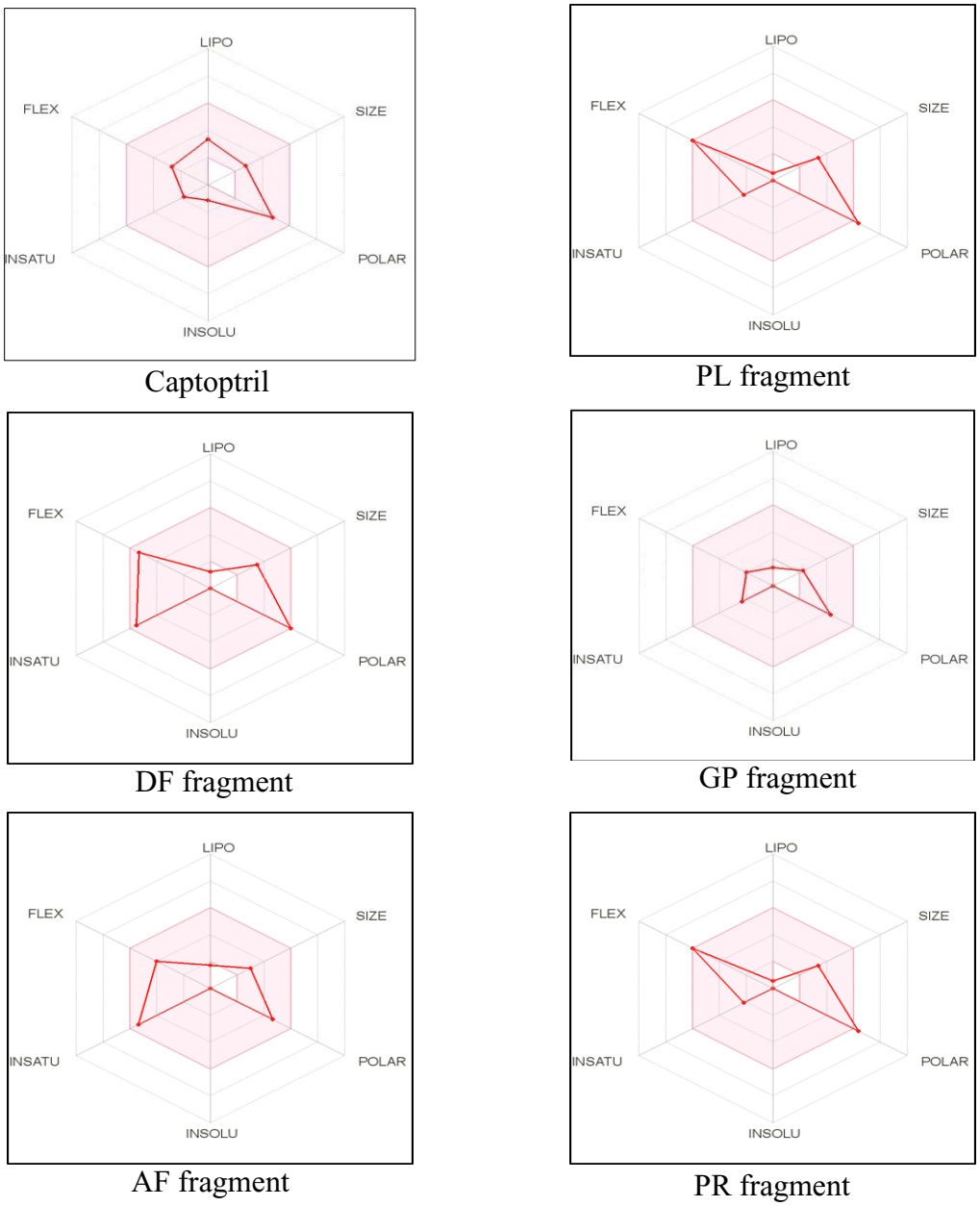

Figure 6.1 Bioavailability radar of sea cucumber-derived bioactive peptide and ACE inhibitory drug (captoptril) based on physicochemical indices ideal for oral bioavailability.

LIPO, Lipophilicity: -0.7 < XLOGP3 < +5.0; SIZE, Molecular size: 150 g/mol < mol. wt. < 500 g/mol; POLAR, Polarity: 20 $Å^2$ < TPSA <130 $Å^2$; INSOLU, Insolubility: 0 < Log S (ESOL) < 6; INSATU, Instauration: 0.25 < Fraction Csp^3 < 1; FLEX, Flexibility: 0 < Number of rotatable bonds < 9. The colored zone is the suitable physicochemical space for oral bioavailability.

6.8 Summary

A bioinformatic approach was used to evaluate the bioactive potential and physicochemical properties of peptides identified from the LC-MS/MS analysis of sea cucumber protein hydrolysates. PepRank was used for virtual screening of bioactive peptides followed by the BIOPEP-UWM database to predict antioxidative and ACE inhibitory potential and simulated gastrointestinal digestion process of selected peptides. The software ToxinPred was then used for predicting the toxicity and physicochemical properties of potent peptides to assess their safety and functionality. Identified active fragment sequences were evaluated for their biological functionality using the web-based application SwissADME, which includes the analysis of absorption, distribution, metabolism and excretion (ADME) of peptides. The listed parameters are crucial in drug discovery process. These predictions elaborated the remarkable importance of peptides derived from sea cucumber proteome as oral therapeutic ingredient. Thus, the findings of *in silico* analysis demonstrated that sea cucumber derived peptides present great potential for the development of nutraceutical products. However, the virtual predictions and simulations should be validated using structure-functional analysis and *in vivo* approaches for confirmation of bioactive properties.

CHAPTER 7

SUMMARY AND RECOMMENDATIONS

This study evaluated the potential of recovering proteins and producing bioactive protein hydrolysates from underutilized Atlantic sea cucumber body parts, including body wall, flower and internal organs. The physicochemical and functional properties of sea cucumber protein isolates were analysed and compared with soy protein isolates, the industrial standard. Leucine was found to be the predominant essential amino acid in the three samples, followed by glutamic acid, aspartic acid, and arginine. Amino acid profiles of sea cucumber protein isolates were in accordance with the recommendations of the World Health Organization /Food and Agriculture Organization (WHO/FAO) 2007 for dietary requirements. These findings indicate that sea cucumber protein isolates can be regarded as rich sources of essential amino acids (EAA). The solubility of the sea cucumber protein isolates, as expected, was pH-dependent. The protein isolates showed a gradual increase in solubility above and below their isoelectric points that demonstrate a typical U-shaped curve. The functionalities of sea cucumber protein isolates were closely related to their solubility, while emulsifying and foaming properties followed the same trend by exhibiting lowest functionalities at the pH range of 4-6, corresponding to their isoelectric points. The water binding and oil holding capacities were determined and compared with those of soy protein isolate. The results suggested that water holding capacity of sea cucumber protein isolates were lower than that of soy protein isolate. In contrast, sea cucumber protein isolates showed higher oil holding capacity compared to soy protein isolates, which can be attributed to their protein composition and surface hydrophobicity.

These findings were aligned with surface hydrophobicity of sea cucumber protein isolates, which possess significantly higher (p <0.05) surface hydrophobicity than soy protein isolate. The hydrophobicity of sea cucumber protein isolates followed the order of body wall > flower> internal organs. However, sulfhydryl groups and disulfide bond contents of sea cucumber protein isolates were lower than those of soy protein isolate, which may be associated with the processing conditions. The FTIR spectra reflected the protein conformity of sea cucumber protein isolates by exhibiting distinct absorption peaks at main wave numbers corresponding to the secondary structure of proteins in all three sea cucumber body parts.

The bioactivity determination of the isolated proteins was carried out by employing enzyme technology. Commercially available food grade enzymes, namely Alcalase (endopeptidase), Flavourzyme (endo- and exopeptidase) and Corolase (endopeptidase) were used to determine the effect of sequential and individual use of endopeptidases and exopeptidases on the production of protein hydrolysates from protein isolates derived from sea cucumber body parts (body wall, flower and internal organs). The highest degree of hydrolysis (DH) from single enzyme-treated samples was observed for samples hydrolysed with Alcalase. Hydrolysates obtained from using endopeptidase and exopeptidase sequentially exhibited significantly higher (p <0.05) DH values compared to that of single enzyme-treated samples in all three sea cucumber body parts. This indicates that sequential use of endopeptidase and exopeptidases could have a higher impact on hydrolyzing proteins compared to that of single enzyme treatments under the same conditions. The protein hydrolysates so produced were then analysed for their radical scavenging and metal chelation activities using *in vitro* antioxidant assays. The radical scavenging activity of the

hydrolysates were evaluated using DPPH, ABTS, and hydroxyl radical scavenging activity assays, whereas metal chelation activity was determined by monitoring their ferrous ion chelation ability. Reducing power of produced protein hydrolysates was also evaluated. A similar trend was observed in most of the *in vitro* assays indicating that samples treated with combinations of Alcalase and Flavourzyme exhibited better antioxidant potential compared to other treatments and their untreated counterparts. As antioxidants play a vital role in food preservation and health promotion, protein hydrolysates were then assessed for their antioxidant efficacy in food and biological model systems. The enzyme-treated samples showed a better prevention of oxidation in oil-in water emulsion systems and comminuted meat model system compared to untreated samples. Analysis conducted with biological model systems, including inhibition of hydroxyl and peroxyl radical-induced supercoiled DNA strand scission and cupric ion-induced human low-density lipoprotein (LDL) peroxidation further supported the fact that the antioxidative potential of sea cucumber proteins was improved upon enzyme treatments. In addition to determination of antioxidative potentials, physiochemical characteristics of protein hydrolysates were evaluated based on their color, solubility and water holding capacity. The efficacy in model systems and the physicochemical properties of protein hydrolysates revealed the potential of using sea cucumber protein hydrolysates as functional food ingredients and nutraceutical for health promotion. Among all enzyme treatments, sequential addition of Alcalase and Flavourzyme exhibited the most favourable antioxidative potential and physicochemical properties in tested body parts of sea cucumber protein hydrolysates, indicating the efficacy of employing combinations of endopeptidase and exopeptidase enzymes. The hydrolysates prepared from Alcalase and Flavourzyme were further investigated for their composition

and potential in ACE inhibition activity. Our findings indicated that Alcalase and Flavourzyme combined treated samples might serve as alternative sources of dietary proteins due to the rich profile of essential amino acids. It was also revealed that samples prepared with Alcalase and Flavourzyme could be beneficial in improving cardiovascular health conditions with blood pressure lowering applications by inhibiting ACE activity.

The protein hydrolysates of sea cucumber body part samples that showed the highest potential were then subjected to LC-MS/MS analysis to determine their molecular weight distribution. Among the identified peptides, over 60% of body wall protein hydrolysate, around 70% of flower protein hydrolysates and more than 80% of from internal organ hydrolysates had their molecular weights in the range of 0.5-1.0 kDa. The identified peptide sequences were further investigated using PEAKS X+ software for their potential antioxidant and ACE inhibitory activities, resistance to GI digestion process, physicochemical properties, toxicity and drug-likeliness by using bioinformatic tools.

The potential bioactivity of sea cucumber-derived peptides was screened using PepRank web-based tool and 0.9 was selected as the threshold value for predicting the probability of a peptide to be bioactive. Among all the identified peptides, 10 (out of 419), 8 (out of 538) and 6 (out of 513) were respectively identified from body wall, flower, internal organs samples as peptides showing higher probability value than the selected threshold value. BIOPEP-UWM data base tool was used to determine the occurrence of specific peptide sequences of selected peptides which had high antioxidant and ACE inhibitory activities. Selected peptides were then subjected to *in silico* proteolysis simulation with representative digestive enzymes, namely pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) using "BIOPEP Enzyme(s) Action"

program to determine their resistance in GI digestion. The resultant peptides after simulated digestion were evaluated using ToxinPred software for predicting their toxicity and physicochemical properties. All digestive resistance peptides (PL (proline + arginine), DF (aspartic acid + phenylalanine), GP (glycine + proline), AF (alanine + phenylalanine) and PR (proline + arginine)) were found to be non-toxic and displayed favourable functional properties indicating their potential to be used in a wide range of food systems, including hydrophobic and hydrophilic systems. Virtual screening of identified peptides was further assessed for their suitability for drug development process by employing robust predictive model of SwissADME. Evaluation of absorption, distribution, and excretion (ADME) parameters of digestive resistance peptides revealed that, excluding PR (proline + arginine), all peptides PL (proline + leucine) DF (aspartic acid + phenylalanine), GP (glycine + proline) and AF (alanine + phenylalanine) had the potential to be used as therapeutics in oral administration.

Considering all these findings, the current study provides a fundamental understanding of using a biorefinery approach for upgrading underutilized Atlantic sea cucumber (*Cucumaria frondosa*) tissues into value-added protein isolates and hydrolysates with attractive functional and bioactive properties with a special focus on antioxidant and ACE inhibitory activities. However, further studies are needed to determine the organoleptic properties of the recovered protein isolates and their structure-functional relationship in association with sea cucumber proteome. Further in-depth evaluation is also required for optimal analysis of the use of endopeptidases and exopeptidases on producing bioactive protein hydrolysates. *In vivo* analysis of protein hydrolysates may further strengthen the findings from *in vitro* bioactive assays. The economic analysis would assist

determining the applicability of sea cucumber protein hydrolysates in foods or supplements in purified or crude forms.

Incorporation of bioinformatic approach to discover the bioactive peptides derived from sea cucumber proteins enabled us to predict the potency of desired biological activities with theorical physicochemical properties. Findings of *in silico* analysis also revealed the pharmaceutical potential of sea cucumber derived peptides attributed to the oral drug discovery process. These findings could further be expanded by studying quantitative structure-functional relationships of peptides. In addition, employing computational tools, including molecular-docking will provide new insights for peptide bioactivity prediction after peptidomic analyses. However, confirmation of all these predictions requires experimental validation. Thus, wet lab synthesis of the specific motifs and related clinical evidence will facilitate the process of providing support for the health claims and commercializing of sea cucumber-derived peptides as a functional food ingredient or supplement or possibly as natural pharmaceutical ingredients.

PUBLICATIONS

Senadheera, T. R. L, Dave, D., and Shahidi, F. (2020). Antioxidant Potential and Physicochemical Properties of Protein Hydrolysates from Body Parts of North Atlantic Sea Cucumber (*Cucumariafrondosa*). *Food Production, Processing and Nutrition* (in press)

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Physiochemical properties of protein isolates from body parts of North Atlantic Sea Cucumber (*Cucumaria frondosa*)

Biological activities of protein hydrolysates prepared from North Atlantic Sea Cucumber (*Cucumaria frondosa*)

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APPENDIX I

[a]Identified peptides from body wall protein hydrolysates using LC-MS/MS

N(+42.01)FSHTNGNSN(+.98)SSGSKSSSSH	TPEGPGDYE
RC(+57.02)GVPQ(+.98)SSLSTSQ(+.98)LPPVSP	YPDGSGPRL
PGNDGGPGPKGEQGDIGPQ	RGPPLGPLGP
SKGFTGPRGDKGPFGPA	LPDDDRTE
DDPDAEKPDDWDDE	DEPIPGSPF
GPLGPM(+15.99)GVQ(+.98)SVVQGYI	PGPNLGKQF
GDPGN(+.98)PGPQ(+.98)GQPGPVGE	PDGKPVQPD
GPFNWRPPLDGPGS	DKPGAPVGPL
Q(+.98)VMSQ(+.98)TERIPNSP	PTPAVVAPTP
GPPGPSGTTGRDGRDG	APEPPPSRP
PGPQ(+.98)PNPPGAFGTLQ	G(+42.01)SDGLPGFPG
MPEEPIYDEPQAG	PGPPGQAGYP
Q(+.98)GPTGLQ(+.98)GLTGPVGSP	GPQSPPPM(+15.99)L
GPPGETGPRGPIGPQ	YPTLPGEY
TGSDYLAVTTTASQ	TPDGKPVQP
GPPGPHSHQTGIDL	Q(+42.01)TGVSGPPGP
GPPGPEGPKGDTGDP	TGPGPRQAGP
MVYVMGEVAHPGA	PAGAPGSPGVAG
DVPEAPSKPSPTE	SPIRIPVGP
RPGPSGPQGAKGDAG	AGQIPPPRP
MPEEPIYDEPQ	FQAGSYGTP
DEGDSGDPGPNGAPG	Q(+42.01)TGPRGDPG
GVPGPPGTAGERGSP	PGIGGPQ(+.98)GNQ
DDDDRAPSPTPE	PGIQ(+.98)GLPGPS
GVPGPQGEGGISGPP	RPGPSGPQGA
DDPDAEKPDDW	STSPGM(+15.99)KSL
G(+42.01)PAGPVQ(+.98)YSRPQ(+.98)	Q(+.98)PPNLPAGE
G(+42.01)APGPEGLRGFQG	IGPKGIPGSP
GPPGASGPLGIAGSM(+15.99)	PGIQGLPGPS
EGSRGEAGPN(+.98)GPR	FMTELGPQ
G(+42.01)PAGQN(+.98)GFPGPSGP	YPEREEP
LGDPGSHGLSALPG	PGPPGPPGEN
GPPGPQ(+.98)GPRGPGTP	PGMIGRQGP
PGVGDPLADGVFE	PAGATGERGP

GPPGEPGPQGPSGH
GPPGASGPLGIAGSM
PGPGGASGNPGLDGD
GPPGPPGM(+15.99)Q(+.98)GPSGL
PGPGNFM(+15.99)MPGPF
PGPGNFMM(+15.99)PGPF
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GPQGPAGQRGPAGP
SPSPMHVGPGPSP
GPPGSSGPAGPPGPN
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GPPGPPGEPGPQ(+.98)GP
GPPGN(+.98)FQGPPSGQ(+.98)
PGPGRSGLQSVGE
GPPGPPGEPGPQGP
VPEAPSKPSPTE
PGPPGKNGRQGAT
PGGPGLGGQ(+.98)Q(+.98)GPRG
TPPVVQNC(+57.02)PTGG
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GPPGAN(+.98)GASGERGP
IPGLGPDPEPEP
QPIMPSPGEEE
GPPGPPGTVQ(+.98)GFT
GPPGPPGTVQGFT
GPPGPQWPLDF
GPPGPKGEQ(+.98)GRK
GPPGSAGSIGALGPA
GPPGPPGPPGPSGQ(+.98)
S(+42.01)GQVLARPDDP
Q(+42.01)GPPGPPGPPGPV
RSEPQRQSHA
GPVGSPGDTGVPGP
GPPGPPRPM(+15.99)VGN(+.98)
GPPGPQ(+.98)GAKGDFG
DNSEPPNQSPT
DDDDRAPSPTP
GPSGLKGHTG
GPQ(+.98)GLQGVPG
GAPGATGPQ(+.98)GP
GAPGATGPQGP
GPPGPKGNQG
GPAGPTGPTGP
GEDHIPGSP
APGKPDPPE
APSTPPPSGP
SPEPEHPL
TPGHGPGPSP
FLSTLTAF
PPAPPPPQ(+.98)P
GGKEIPNSP
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PATGPGPGRS
PDVVVPGN(+.98)P
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APPVPPPPN(+.98)
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YPQDPPR
SPRSLPVD
GPEEPQVN
QDAPVPGSP
APGAPSPGNV
GPPGLPGRD
GPRGPPGIN
GPRGPPGEP
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TPAC(+57.02)IPTP
GSPGRPGLD
TPSGNSVPP

EGDDGPDGTPGPA	APGERGDPG
GPVGNRSHPSGL	RPLEPIE
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C(+57.02)(+42.01)HIIKPAYE	GPRGPPGSE
NVSVAHAFN(+.98)PN	TPPANADAP
PSGAPGAPGERGD	TPAVVAPTP
SPIRIPVGPET	RPGPSGPQG
TPEPQPGNPKP	NPGPQGQPG
Q(+.98)GVGGPRGASGPD	EDHIPGSP
GPAGDRGQLGAPG	APGARGHKG
AGSMGPRGPPGAP	YPDGSGPR
G(+42.01)PAGPPGPPN(+.98)FT	PGRPGLPGP
TLMN(+.98)IPFESP	TPGPAGPPGP
P(+42.01)NFGPPGPPMP	PDDDRTE
GPPGEPGPPAGPD	PTNFPDR
TPQGKGPLPEI	DDETPRL
GPQGPQ(+.98)GDLGLP	TPSRASEP
RGPAGPTGPTGPA	PGPPGQAGY
PGQ(+.98)PGRDGPPGP	QPIPGSPF
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GPPGPQ(+.98)GAKGDF	GPRGPN(+.98)GSP
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GEAGVPGEEGPQ	GPVGPQGPE
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G(+42.01)PQ(+.98)GAAGQ(+.98)NGAPG	GLPGPLGPE
G(+42.01)PAGQ(+.98)Q(+.98)GPSGPQ	PGLGPIPGE
EGPIHKDEPV	PGKPDPPE
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GPGGPPPPDGPAP	TPEGPGDY
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EPGRN(+.98)GDPGLP	S(+42.01)VPAYRP
EGPAGQ(+.98)PGIGPE	LPGGPPPPP
ETGPRGPIGPQ	PLPGGPPPP
Q(+4+A111:B1522.01)GPQ(+.98)QPQ(+.98)GQP	IPNSPVTT

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GPGPSPSPGPGPG
Q(+.98)GDGRGGQ(+.98)GPQ
TPSSPWFHP
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PEEPISP
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IPGSDGLP

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GPSGPPGPA
FPGNAPQ
IPNSPVT
LPNSPVT
IPGPDPQ
LPGPDPQ
DVPGSPF
IDQPGST
DEGPIGQ
SDPVPAE
GPSEPVE
IPVGPET
GLPGPLGP
GIPGPLGP
PGLGPIPG
GLPGLPGP
GLPGIPGP
GPGTDYP
PEGPGKD
IPGSDPL
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PAGPSGPP
DPTGPLA

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S(+42.01)SSASQRSE
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PGPRGEPGDP
SLVTSPNYP
PNYPARYP
PEFLFEPP
TPGAMQGIAQ(+.98)
TPIPSDSTR
DDVIIAPID
IPIDDEAPT
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LGGQ(+.98)QGPRGP
Q(+42.01)PTNPRPD
EQRGPPGPQ
GPAGQ(+.98)RGPAGP

DPTGPIA
NQ(+.98)PGPGP
GPGGPGAPG
PQGPPGL
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LPGDVGP
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GPATGPGP
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LPGPLGP
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GPGMMGP
PTGGITP
GPAGPSGP
PVGAPGE
GPAGGPY
PGGAGYP
GVEGPPG
PGVPGSP
PAGPIGP
PGAPVGP

[Letters inside the parentheses indicate posttranslational modifications including (+42.01): acetylation; (+15.99): oxidation; (+0.98) deamidation]

[b]Identified peptides from flower protein hydrolysates using LC-MS/MS

GPAGPTGPTGPA
EGPRGPPGSE
EGPRGPPGSEGR
TPDGKPVQPD
RPGPPEDL
SDEDNLDDVIIAP
SPIRIPVGPE
NPYDEPGMPGVPE
KEDDDDRAPSPTPE
IDDPDAEKPDDW
NPDDPPTWK
VDKPGAPVGPL
SGPAGPPGPNGIDGSQ(+.98)VKM(+15.99)
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TPDGKPVQPDIVDN
GPAGPTGPTGPAG
GPAGPTGPT
GPAGPTGPTGP
GEDHIPGSPF
DDPDAEKPDDW
DDDLPPILN
FDDDLPPILN
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VEGPEEPQVN
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PRGPPGSEGR
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PGPQGIGGPGP
GVDNPGHML
GDRGFPGA
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TVEYM(+15.99)PTK
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TDVPNEGR
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RQGPNQ(+.98)GMGPK
GPPGPQWPLDF
KPTAPGI
GPRGEPGFP
GQ(+.98)RGPAGPTGPTGPA
PGQ(+.98)QPLGPQ
PIDDEAPT
FDPVIEE
GPPGPNGIDGSQVKM
DDGGVPIT
PEEHPVL
Q(+.98)GPQQAGIPGLMQQ
FTITYLAP
APIYDEL
GPEPGLVPQ
GAPGPRGNEGSQ(+.98)G
DDPTVPR
PQ(+.98)N(+.98)N(+.98)DEKEP
GLPGIPGPA
IGPIGPT
GPVQYSRPQ(+.98)Y

M(+15.99)DYDERPN
GSPGPRGPN(+.98)GSPGSQGQ
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G(+42.01)PAGPAGPT
DKGEQGPPGPSG
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PNSPVTTK
A(+42.01)PGGPGGPGYAQ
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TPIPSDSTR
LPGDVGPQ
EDHIPGSPF
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IPIDDEAPT
ADEPIPGSP
DVPEAPSKPSPT
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GPTGPTGP
SVGEPGDPGQVGSP
GLPGDVGPQ
GVDNPGHPF
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SDPVPAE
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EVGN(+.98)PLKIK
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KYDFGPI
TPSIGRPLAN
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GEMPVDF
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PRGPPGPSGTTGRD

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DVPGSPF
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SPRSLPVD
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QDPPAQPY
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KPIYTPD
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VPSPDYE
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YPTLPGEY
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DEPIPGSPF
GPRGPPGSEGR
Q(+.98)GPVAPVGP
Q(+42.01)RGPPGPQE
GGPGGPGAPG
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A(+42.01)GESPSKNGAD
DGTPGPAGRQGE
KAAPARHQ
GPSGPQ(+.98)GAKGH
VPDLPLSQ

DEEDIDKSP
TPHITPT
RPLEDVE
GIHVPGSP
GEATLPIGR
PLGLTGRPGP
TSPGSDGN(+.98)GQ(+.98)RQ
EGPRGEPGFPGSN(+.98)GPAG
Q(+42.01)QGPRGPPGPSG
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GQPRPGMP
DKPVSPL
LKDPLEALP
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GDEGDSGDPGPN(+.98)GAPG
DKPGAPVGPLEPS
YPTVPGE
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RPDIVDPA
GPGGPGPGM
PAGPSGPP
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DDDDRAPSPTPE
KPGAPVGPL
DEPIPGSP
GFPEMPPQPPVIR
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GAPGARGHKG
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RPGQEYE
GDKGEQGPPGPS
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GPLGLTKYMV
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PGDVGVE
K(+42.01)TMTPGK
GIQGPQGPR
Q(+42.01)SVPHEGI
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DSDDVPSF	TPHPKLEPP
GPRGPPGSE	TVGTPVDI

[Letters inside the parentheses indicate posttranslational modifications including (+42.01): acetylation; (+15.99): oxidation; (+0.98): deamidation]

[c] Identified peptides from internal organs protein hydrolysates using LC-MS/MS

IDDPDAEKPDDWDDE	DNTEPPY
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MPEEPIYDEPQAG	PDTLYPQ
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NPYDEPGMPGVPE	PDEIRDS
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DDPPEPPLHNGPT	RPLHPNP
DVVVNPPQKGEPS	SPSIPVTE
MPNDDITHPIPD	SPSPVVDQ(+.98)
E(+42.01)APQ(+.98)ETPHITPT	IPNSPVTT
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EDVTPIPSDSTR	TPETPVPS
M(+15.99)M(+15.99)DYDERPNN	PEDQ(+.98)LPQ
DDPDAEKPDDW	APIIVQPS
EGLELPEDEEE	SPDDVYE
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SHPIEVPVGHPD	NPYGLPY
FPKDDPTKPVH	DLPDGGHL
PGFFWDEIYP	Q(+.98)GPVAPVGP
VDWADPIIEPD	DPIFFPS

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QPDGQMPSDK
GDSGDPGPNGAPG
TPPLQPPRGE
PQ(+.98)SRGQPHSP
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VPSPDYE
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NPDFDPT
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S(+42.01)STTLPR

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SDTVVEP
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PYGNAPQ
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DIIPGTE

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RDFPDGR
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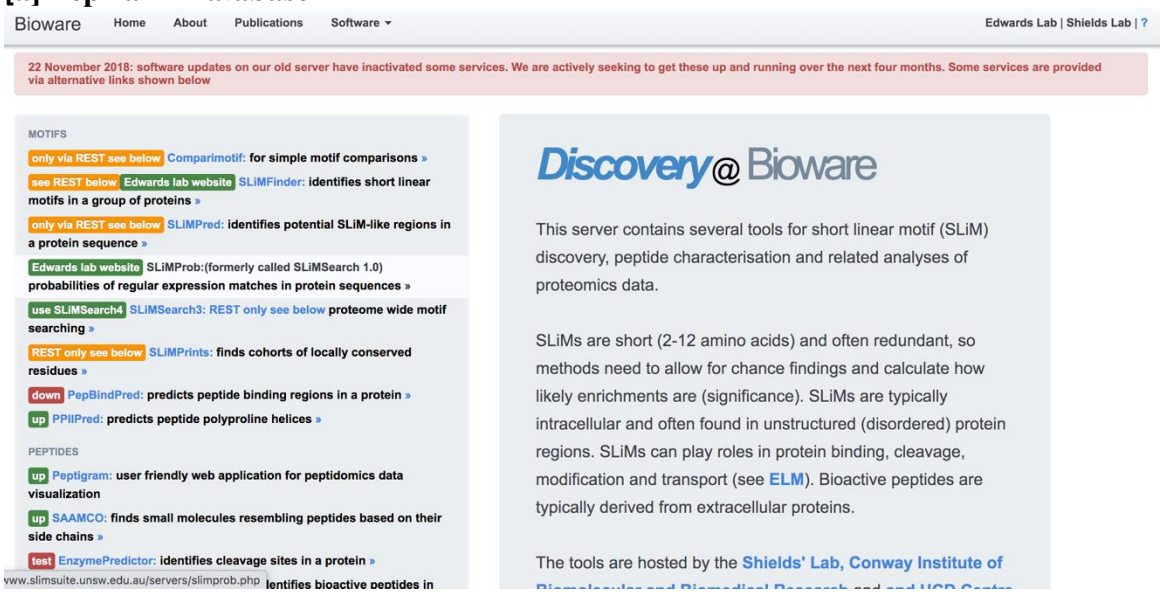
GPGGGIP
PGGPGAP

ELNPEHP TAGGGGI
TPEGPGDY

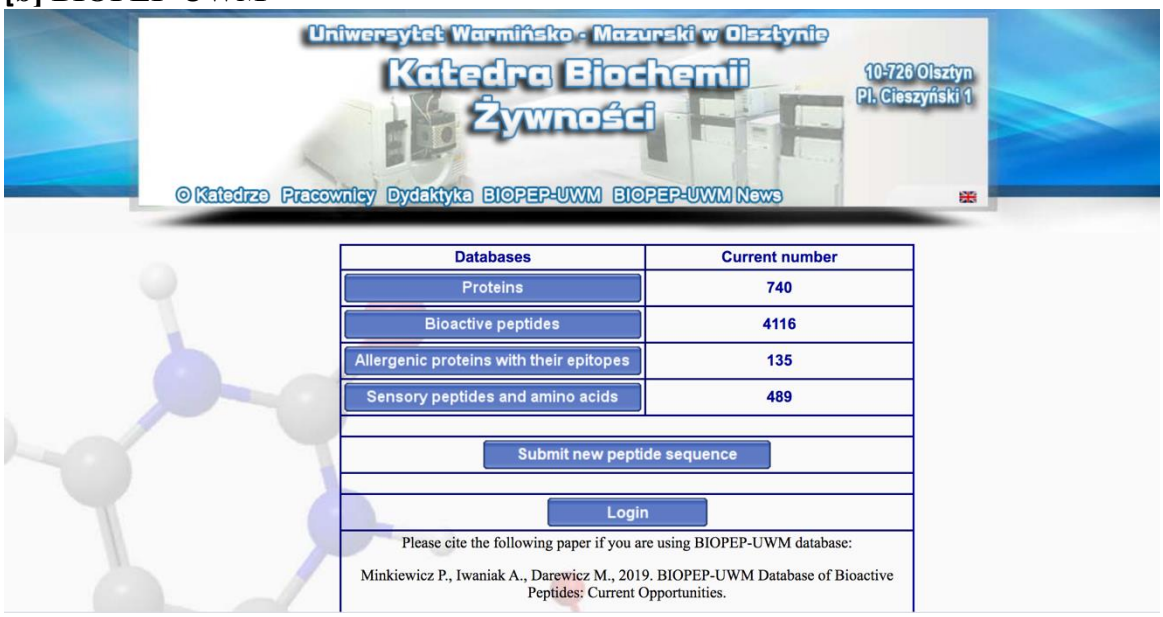
[Letters inside the parentheses indicate posttranslational modifications including (+42.01): acetylation; (+15.99): oxidation; (+0.98): deamidation]

APPENDIX II

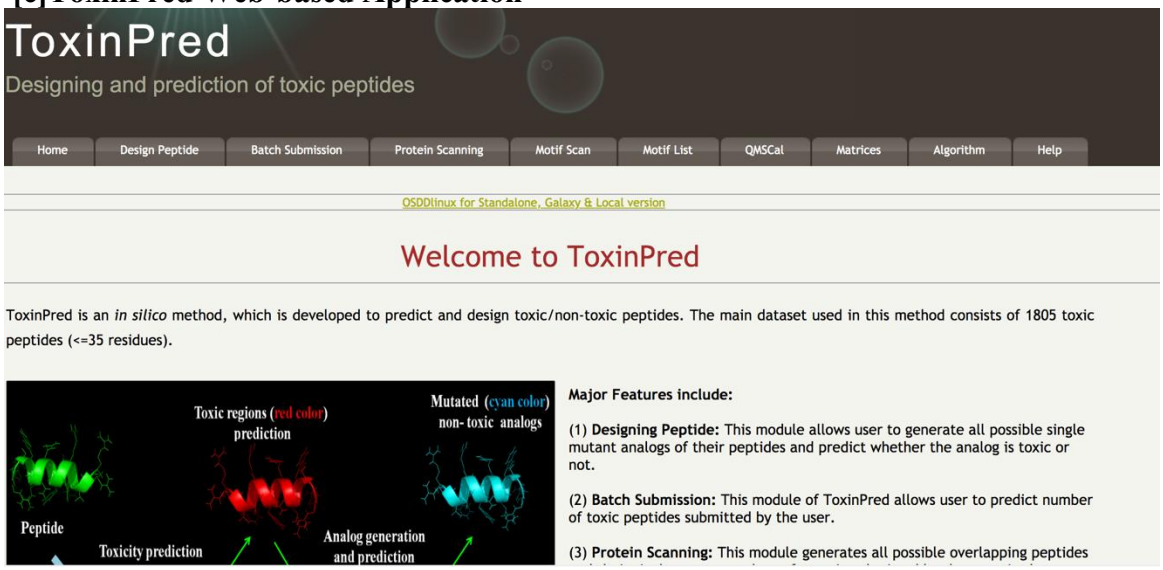
[a] PepRank Database



[b] BIOPEP-UWM



[c]ToxinPred Web-based Application



[d]SwissADME Web-based Application