Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Abdulbasit Aliyu

Biochemical Composition and Bioactive Properties of Mesopelagic Fish-Derived Protein Hydrolysate from Northern Krill (Meganyctiphanes norvegica) and Mueller's Pearlside (Maurolicus muelleri) for Possible Utilization of Novel Marine Resources

Master's thesis in Biotechnology (MSBIOTECH)
Supervisor: Ida-Johanne Jensen
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NTNU
Kunnskap for en bedre verden

Preface

This thesis was written in partial fulfillment of Master of Science degree in Biotechnology (MSBIOTECH) at Norwegian University of Science and Technology (NTNU). This thesis was carried out as part of PhD research of Dat Trong Vu, and will be included during the publication of his research paper. The thesis was carried out under the main supervision of Associate professor Ida-Johanne Jensen at the Department of Biotechnology and Food Science in Akrinn, Kalvskinnet, and Professor Turid Rustad at Department of Biotechnology and Food Science in Gløshaugen. Major part of the experiment was conducted at Akrinn, Kalvskinnet, and a few of the experiment were carried out at Gløshaugen.

My sincere appreciation goes to my main supervisor Ida-Johanne Jensen who has supported me throughout the entire process, and whose immense guidance and suggestion has help me to developing valuable practical and problem-solving skills in Analytical Chemistry and Food Processing. Furthermore, she provided emotional support, and encouragement which aided the smooth transition and timely completion of my practical work. Her valuable suggestions, corrections and recommendations which ultimately assisted me in thinking deeper to write relevant information is outstanding. Also, the communication platform she devised which ensures smooth interaction had helped me a lot. I would like to extend my gratitude to my co-supervisor Turid Rustad who provided valuable suggestions and advice while working at Gløshaugen, and who had also given valuable suggestions during my result compilation, free and total amino acid analysis.

My appreciation further goes to Dat Trong Vu, who had taken me through the step-by-step of each experiment method and who profoundly helped guide me in data analysis using Excel. His effective, timely response and communication throughout the entire procedure and his contribution in the correction of my materials and method contributed a significant portion towards the success of this thesis. Furthermore, his contribution towards designing my master thesis plan gave me a rough overview and further guided me during the experiments.

I would like to thank my parents; my dad, late Alhaji Najimdeen Ali, and my mum Alhaja Latifatu Ali whom their positive inclination towards education orchestrated my academic success right from high school till now. My profound gratitude goes to my uncles; Lawal Abdullah, Lawal Kamal, and Lawal Abbas Hashimiyu who had given me the platform and funded my graduate education to Norway. Your guidance, moral and monetary support throughout my master's degree had shaped me and served as a pedestal to attaining greater success in my career in the years to come.

Trondheim, May 15th, 2022

Abdulbasit Aliyu

Abstract

The growing world population comes with a serious concern on how to sustainably meet the people's nutritional demand now and for the generations to come. The double burden of disease which is a surge in communicable and non-communicable disease, which is further exacerbated by globalization in the 21st century has made the sustainable future not forthcoming. Significant effort to address this problem as part of the sustainable development goals is required to intensify. In this regard, the ocean has been identified to be a good source of protein containing food which can potentially solve the world food requirement through sustainable aquaculture and development of novel technology. New marine fish resources of the mesopelagic zone contain vast amounts of biomass that can be exploited to support the regular aquaculture. Protein hydrolysate of these marine mesopelagic fish has also been argued to be exerted antioxidative and antihypertensive inhibitory attribute. This, therefore, longing for new research to establish and refine the position of marine mesopelagic fish in the sustainable future.

Biomass containing two and one mesopelagic fish were processed and investigated in batches. Raw materials sample were subjected to enzymatic hydrolysis, and the recovered samples were filtered and freeze dried to a finely refined protein hydrolysate powder. The aim of this thesis was to study the nutritional composition of these mesopelagic fish protein hydrolysate, by conducting the proximate composition, evaluate the antioxidative activity, and antihypertensive activity. Also conducted molecular peptide sequencing to know the molecular weight of peptides fraction after membrane filtration. Furthermore, to determine the degree of hydrolyzation, and to examine the yield of the protein hydrolysate and discuss possible optimization.

Protein hydrolysate from both batches displayed high protein, and other constituent were within expected range described for marine fish. The protein hydrolysate showed high content of lysine, leucine, glycine/arginine and, and glutamate. The antioxidative capacity was shown high when measured by FRAP and ABTS, but low when measured by ORAC. The result of the sample containing only pearlside were extremely low compared to the sample containing both krill and pearlside. However, there is a need for pre clinical and human clinical trial to verify these results and establish safe dosages. The molecular weight distribution has shown high content of smaller peptides < 2kDa in all protein hydrolysate. With the abundance of lysine, leucine, and glycine in the free amino state. di-, tri-, tetra-peptides containing these essential amino acids in any order are suspected to be responsible for the antioxidative and hypotensive effect. Alcalase base hydrolysates from both batches has shown highest ACE inhibitory effect, although there is a need for further refinement in the procedure. The degree of hydrolyzation and yield have shown no significant different between enzyme-based hydrolysates.

Result from this thesis corroborate the developing knowledge on the therapeutic and nutritional attribute of the marine mesopelagic fish. Based on the yeild, degree of hydrolyzation, and bioactivity, endogenous enzyme are very promiscing and can potentially be tuned for commercial application

Table of Contents

List of Tables

List of Figures

Glossary

CVD	Cardiovascular disease
ACE	Angiotensin I-converting enzyme
DVM	Diel vertical migration
PUFAs	Polyunsaturated FAs (fatty acids)
FPH	Fish protein hydrolysate
UN SDG	United sustainable development goals
CMNNDs	Communicable, maternal, neonatal and nutritional diseases and injuries
NCDs	Non-communicable diseases
ROS	Reactive oxygen species
ORAC	The oxygen radical absorbance capacity radical cation
ABTS	2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonate
FRAP	Ferric reducing antioxidant power
DPPH	2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl radical)
RAS	Renin-angiotensin-aldosterone system
DH	Degree of Hydrolysis
HPLC	High-performance liquid chromatography
TE	Trolox Equivalence
PG	Propyl gallate
AAPH	2,2'- azobis-(isobuttersa ureamidin) dihydroclorid
AUC	Area under the curve
FAA	Free Amino Acids

1 Introduction

The advent of the twenty-first century has seen an increase in the world population [1], yet the world population is expected to increase by 2 billion individuals in the coming 30 years, ascending from the present 7.7 billion to 9.7 billion, and reach a peak of 11 billion in the year 2100 (Figure 1) [2]. This population increase has posed a serious question on how to feed the growing masses so that everyone gets the required amount of nutrient necessary for the body functioning and without traceable ecological footprint [3]. Food production needs to increase by 70% in other to meet the world demand in the coming 30 years [4], sadly, with the burden placed on the terrestrial resources, evident in growing greenhouse gas emission, decrease in agricultural land, loss of biodiversity, compromise of the terrestrial ecosystem, and limited water resources, propagation of popular terrestrial farming will leave so much impact on ecosystem. Furthermore, challenges like decrease rate of terrestrial biomass yield, has affected the land-based effort in meeting world nutrient demand. This calls for sustainable and alternative means of food production with the goal to meeting the UN sustainable development goals, therefore, it has been encouraged to investigate the sea for more food due to it tendency to be more sustainable and to ease the tension placed on terrestrial food chain while sourcing for protein. Seafoods may contain bioactive chemical compounds which are rarely found in the land-based foods [5], indicating a promising option to contributing significant quota to global nutrition, serving as functional food and nutraceuticals [6]. Meat has a higher carbon footprint compared to, seafood, which is more sustainable and can be termed as being "carbon friendly" when explored in a sustainable manner [7]. As of today, seafoods account for 17% of the total edible meat protein produced globally, and has the potential to increase by 36 – 74% resulting in 21– 44 million tons, and 12 –25% of meat expected to feed 9.8 billion people in 2050 [6].

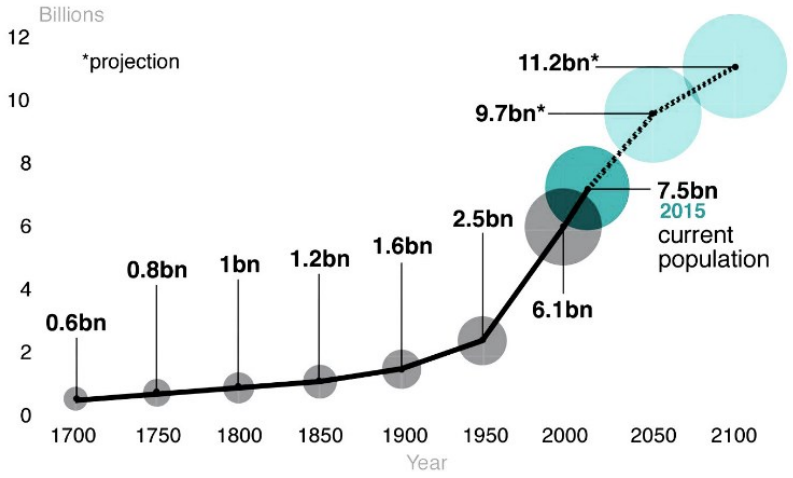

Figure 1: Progression of world population growth echoing the need to increase the world food production by 70% in 2050 [4].

In this light, it is important to stress the UN (United Nations) sustainable development goal 2 and 3 of the 2030 agender "end hunger, achieve food security and improved nutrition and promote sustainable agriculture", "ensure healthy lives and promoting well-being for all at all ages", [8]. Rapid urbanization, and increased income accompanying the emerging economy had led to increase in demand for more proteins and a quest for sustainable source. It became imperative to source alternative food source and ingredients, more emphatically focused on proteins, and marine fish is a promising source. However, hampering the effort towards realization of the UN sustainable development goals, is the significance of the cardiovascular disease (CVD) which has been the major cause of death globally, accounting for 46% of death related to non-communicable disease [9].

The lower trophic level which houses the mesopelagic zone (twilight zone) extending from 200 to 1000 meters depth, constitute 60% of the earth surface, 20% of the ocean volume [10]. It contains a robust amount of biomass ~1,000 million tons and accounting for a larger percentage of the world total fish biomass (Figure 2) [11]. Proposed global mesopelagic fish of ~10,000million tons is extremely large compared to annual traditional fisheries of order ~100million tons [12]. The all-encompassing mesopelagic species of fish can potentially be a source of nutrient to meeting human daily nutrient demand, the UN sustainable development goals, and serve as alternate feed sources for the rapid development of aquaculture [13].

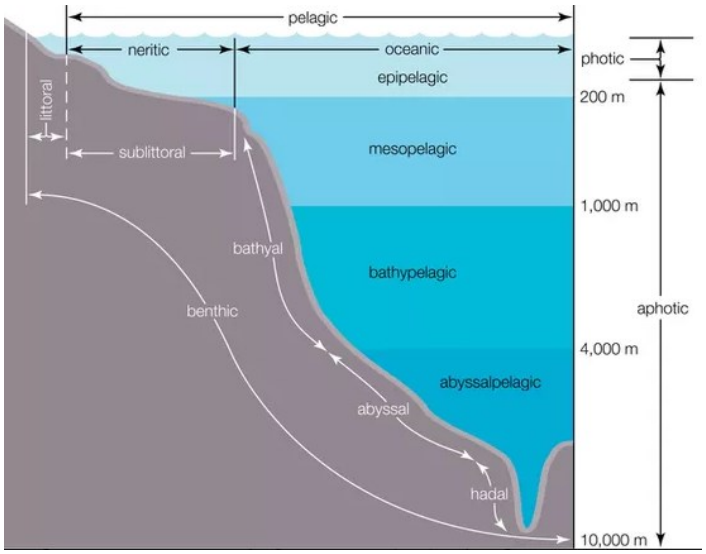

Figure 2: Ocean zone showing the mesopelagic zone with depth ranging from 200-1000 meter, just below the pelagic and epipelagic zone [14]

Sequel to the foregoing, researchers have channeled recent research to focus on marine mesopelagic fish species due to increasing demand of proteins, marine fats, and sustainable feed source for aquaculture [15]. Recently, significant interest has been placed on the bioactive peptides present in marine mesopelagic fish protein hydrolysate, this seemingly unique peptides may have considerable and promising therapeutic effect (Figure 3). Interestingly, clinical intervention and animal studies have attributed the metabolic health benefit of fish-derived peptides to taurine, although with yet to established mechanistic proofs, and conflicting literature reviews [16, 17]. Their structurally diverse bio-composition resulting in possible higher bioactivities "the structure-function relationship" can be trace to distinctive light, temperature, and pressure conditioning in this ecological zone, coupled with the location of a particular organism in the marine ecological food chain [8].

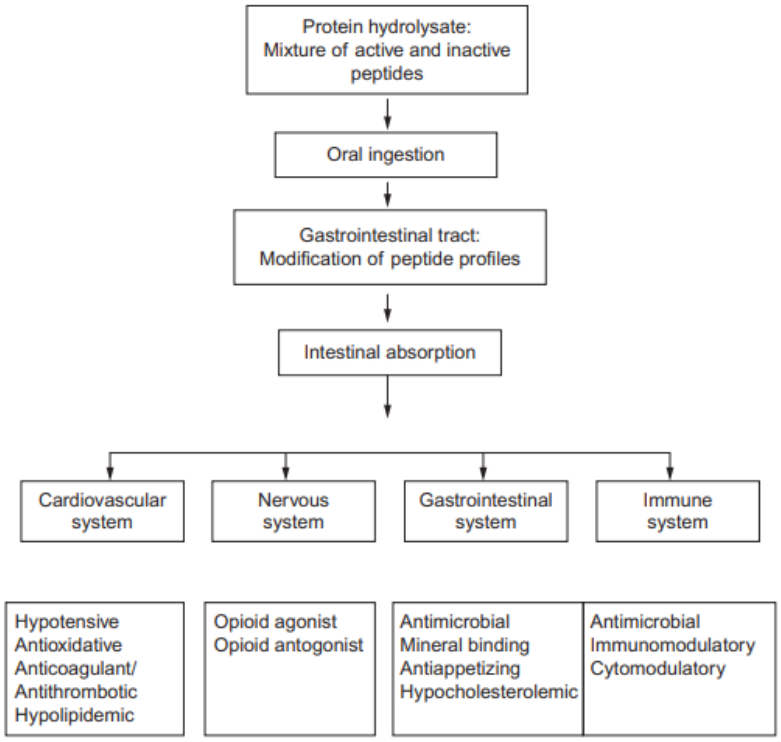

Figure 3: Physiological effect of bioactive peptide depicting various system and effect particular to it [18].

Bioactive peptides are naturally occurring compound or protein parts which are inactive in their precursor but active after hydrolysis and can be recognized by their specific receptors in the cells [19]. The hydrolysate from marine fish may contains biological active peptides or amino acid which possess antioxidant, antimicrobial, anti-inflammation, anti-ageing, anti-hyperlipidemia, anticoagulant, chelating, angiotensin I-converting enzyme [ACE] inhibitory effects, and type 2 diabetics regulation (Figure 3) [16, 20]. However, with the abundant of marine mesopelagic fish

and the possibility of improved structurally diverse-induced bioactivity characteristics present in their hydrolysate, limited research has been done to explore this untapped resourced in producing protein-rich functional product, and production of improved bioactive hydrolysate capable of curing the cardiovascular ailment [8].

1.1 Aim of the thesis

The overall aim of this thesis was to characterize and compare the biochemical composition of two different batches of marine mesopelagic fish and investigate the *in vitro* bioactivities of the protein hydrolysate made from different food grade enzymatic hydrolysis.

The overall aim was further differentiated into the following sub-goals:

1. Compare bioactivities obtained from two different batches of mesopelagic fish with their respective dried protein hydrolysate powder, antioxidative capacity, effect of hydrolyses and enzymes
2. Compare the yield of the protein hydrolysates in both batches of raw materials and investigate how different enzymatic hydrolysis affect this yield, free amino acids, molecular size distribution and the bioactivity of the protein hydrolysate.
3. Characterize the protein hydrolysate and investigate the relationship between molecular peptide size (kDa) and bioactivity.
4. Investigate the angiotensin converting enzyme (ACE) potential of the two batches of the protein hydrolysates and compare the activity of different enzymatic hydrolysates.

1.2 Marine mesopelagic fish

The mesopelagic zone is known to harbor the highest population of marine vertebrate fish species in the world. The mesopelagic fish species are diversified, and the population varies between 200m and 1000m depth (Figure 2). The variation in population across this zone is affected by intensities of the light reaching it. Important attributes of these novel fish species are diel vertical migration (DVM), which is the characteristic to-and-fro movement between the lower depth at the daytime and the ocean surface at the night, modulated by the availability of food, this phenomenon is important for the biological carbon pump. Marine mesopelagic fish play an important role in the marine food web, they mostly feed on zooplankton and are prayed on by pelagic fish and sea birds [21]. Ocean current and advective processes are two natural forces identified that modulate the distribution of the mesopelagic biomass. Nutritionally, marine mesopelagic fish is a potential source of polyunsaturated FAs (PUFAs) and high nutritional value proteins. Omega 3 FA from marine source is of important health benefit [22], alternate rich source of this acid will ensure it production meet the health requirement [23]. Results from analyses conducted on the mesopelagic fish harvested in Norwegian fjords has shown that the free amino acids (FAs) contain 30% of omega-3 FAs. Additionally, mesopelagic fish are rich in protein, lipids, and bioactive compounds. [24]. Marine protein is well documented to contains all essential amino acids such as leucine which is needed for the building of the body tissues and can also be used in development of drugs or

protein supplement [25]. Furthermore, mesopelagic fish peptides has been shown to plays an interesting role in modulation of cardiovascular diseases via their unique anti-oxidation and enzyme inhibitory activities (Figure 3) [26]. The mesopelagic fish has an estimated biomass of 6-10 billion metric tons globally, however, there is little knowledge about their biology and ecology, the knowledge gap hinder how scientist can sustainably harvest and use this biomass. Furthermore, spatial-temporal knowledge about how mesopelagic fish is distributed is important to sustainably harvest them. Currently, there are challenges in the method developed so far to study the distribution of marine mesopelagic fish, this bias must be overcome for effectively exploitation and without ruining the biodiversity of the oceans [24].

1.2.1 Mueller's pearlside (Maurolicus muelleri)

Mueller's pearlside (Figure 4), is a small fish with size 4-5cm and have a short lifespan of 5 years maximum, with maturation period of one year. This fish species is found mostly in the continental slope and Norwegian fjords, although it has also been said to be separated regionally with different species. Compare to other fish that harbor this region, Mueller's pearlside has significant population in the upper mesopelagic zone. Mueller's pearlside are regarded as planktivore, feeding on phytoplankton and zooplankton, and are also source of food for higher piscivores in the food web. Mueller's pearlside has a prolong spawning period which start in March and end by September in Norwegian fjords. The prolong spawning period coupled with large geographical distribution give the fish the attribute to withstand the instability of hydrographic condition [27]. Mueller's pearlside plays an important role in the transmission of energy between meso- and epi-pelagic trophic food web. There is no report on horizontal migration of this fish, and this is a good indication to potentially form local stock within the fjords. However, they have a unique vertical migration pattern that varies with season, and light exposure. There exists a variability in the phenotype of Mueller's pearlside, and this is because of modification in available resources, predation, and inherent genetic difference accumulated during the evolutionary event. There is little knowledge about the pearlside population biology, and genetic studies is very promising in shaping this landscape [28].

Figure 4: Pearlside picture showing bioluminescence phenomenon [29].

1.2.2 Northern Krill (Meganyctiphanes norvegica)

The word krill derived from Norwegian *kril*, meaning very small fish fry, plays important role in marine ecology and has a pronounced commercial implication yet to be fully exploited. Northern krill (Figure 5) contribute significantly to the euphausiid community biomass by playing a key role in food web cycle, serving as consumer of organisms at the lower trophic level and food for larger predator. In addition to its ecological role, Northern krill biomass also influence the benthic and epibenthic by serving as a source of biogenic materials to these communities [30]. The exploitation and uses of Northern krill fish has developed over time. The major potential use is in the formulation of alternate feed for salmon aquaculture based on new promising protein source that might sustainably support the growth of aquaculture industry. To develop a sustainable feed meal from krill, it is important to exclude the exoskeleton from medium due to it toxic fluoride content (Figure 5) [31, 32]. Another promising research on krill is the development of dietary supplement such as krill oil, which has been documented to aid the treatment of cancer, immunodeficiency, and nutritional disorder [33]. The list of health benefit associated with krill is growing [33]. The krill polyunsaturated omega-3 fatty acid (about 19% of total fatty acids) obtained from krill has a unique stability that allows the oil to be stored for longer time [34]. Chitin and chitosan produced from krill are used in the development of cholesterol lowering drugs, and anti-inflammatory drugs that aid in wound healing [35]. The major innovation by which krill can be harvested has to consider the *in vivo* activities of the biochemical constituent to be extracted. This require the krill to be caught fresh (alive), in good condition and ensure that the krill has not undergone stress which can hinder the bioactivities of the extracted hydrolysates. While harvesting, the dispersed demographic distribution of northern krill within the zone of the oceans has made it difficult to achieve the criteria of krill being in good condition after harvesting, this condition can be appropriately meet when operating at a local scale processing. However, the means of catching large biomass of northern krill for commercial exploitation need to be developed to contributing to food security [30].

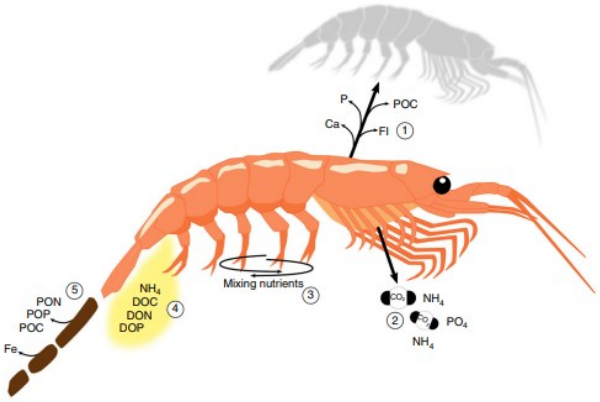

Figure 5: (1) Picture of Krill showing nutrient circulation and a portion of exoskeleton to reiterate the fluoride content [36].

1.3 Processing of protein hydrolysates

There are several approaches that have been developed to extracting fish protein, and depending on the usage of the hydrolysate, parameters such as pH, temperature, weight-to-volume ratio, the number of sequential extractions, agitation time, and homogenization are usually factored in the process. Chemical process was the first approach used to produce protein hydrolysate. This method utilized pH of the medium together with high temperature (121°C) and pressure (100kPa) to cleave fish protein into peptides of different molecular size [37]. pH shift approach has been used to precipitate protein from fish based on the pH and isoelectric point. The surimi process can also be used in the extraction of protein in stepwise manner, this water-based approach involves washing and subsequent dewatering the minced fish in other to remove impurities. This approach is less effective compared to pH-shift as the gelation ability and the product quality is greatly hampered, and due to loss of sarcoplasmic protein in the process, consequently lowering the protein yield [38]. These approaches to produce protein hydrolysate are less effective considering the functional, physicochemical, and bioactive attribute of the final protein. Since the aim is to produce high quality protein hydrolysate with wide commercial application, a more refined approach to produce protein hydrolysate is needed. The enzyme-assisted approach (Figure 6) as used in this thesis involved the usage of food-grade proteolytic enzyme to hydrolyze the protein in the raw materials; this process also utilized the intrinsic attributes of enzyme such as selectivity and specificity to yield an optimal product.

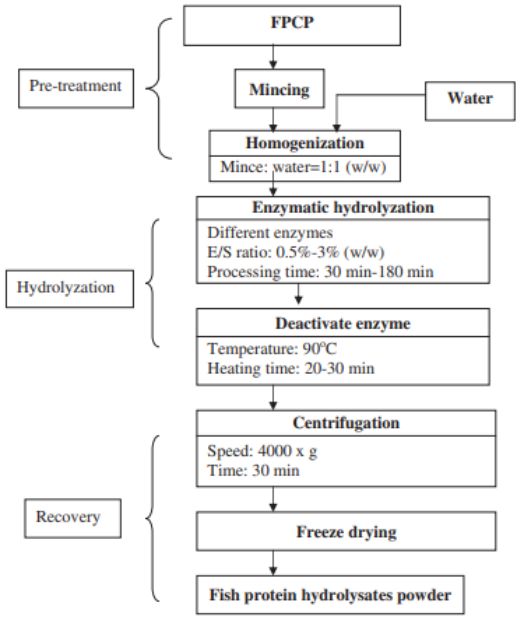

Figure 6: Step-by-step overview of enzyme-based approach in the production of protein hydrolysate [39] [20].

1.3.1 Enzyme for hydrolysis

Sustainability of fish protein hydrolysate (FPH), to foster the UN sustainable development goals (SDG), the enzymatic modification (Figure 6), which has shown an increased techno-functional, physicochemical, bioactive, and organoleptic while retaining the nutrient value of the product is the most practical option [40]. In human, the dietary protein from food is broken down in the gastrointestinal tract, and then absorbed in the form of small peptides (di- and tripeptides) [41] which are absorbed faster than the free amino acid into the system [42]. The protein can either be cleaved (Figure 8) by endogenous enzyme present in the fish or via addition of commercial enzyme. Proteolytic enzymes are classed based on their properties, such as whether they are endo- or exo-acting proteinases/peptidases. Endo proteinases, for example, cleave peptide bonds within the protein to release peptides or shorter fragments, whereas exopeptidases cleave terminal peptide bonds to extract single amino acid residues or tiny peptides from the C-terminus (carboxypeptidases) or N-terminus (aminopeptidases) (Figure 7) [43].

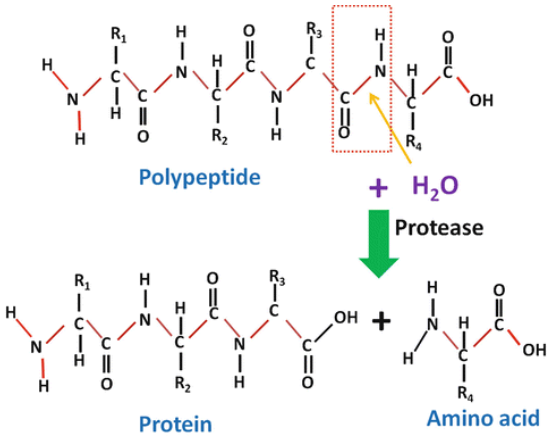

Figure 7: Hydrolysis of polypeptide with protease in an aqueous medium to produce protein and amino acid [44].

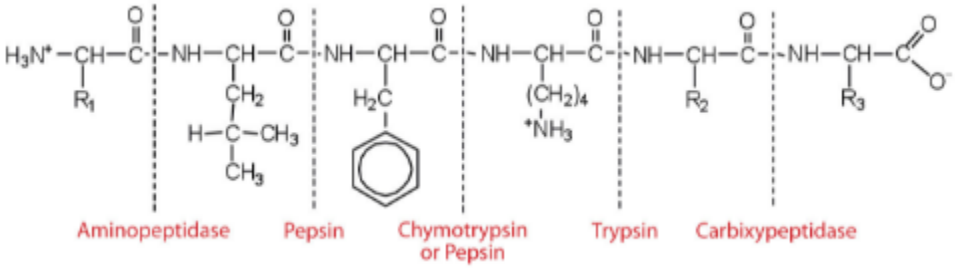

Figure 8: Positional illustration of peptidases action sites on polypeptide chain [45].

The endogenous enzymes used in control experiment are intrinsic proteins found in gastrointestinal tract of fish, while some such as calcium-dependent proteinases, cathepsins, collagenases, and alkaline proteases can be found in the fish tissue. Although with considerable activity, endogenous proteolytic enzyme found in fish has lesser activities when compared to those found in terrestrial animals [46]. Several food grade products have also been developed either from bacterial or plant source, example of such are Alcalase (*Bacillus licheniformis*), Neutrase (*Bacillus amyloliquefaciens*), bromelain (*Ananas comosus*), papain (*Carica papaya*), chymotrypsin, pepsin, and trypsin [47]. As a result, when carried out in a controlled environment with the use of thermal treatment for enzyme inactivation, enzymatic hydrolysis with exogenously added food-grade proteolytic preparations represents a viable approach for the generation of bioactive peptides from marine mesopelagic fish for consumption and development of functional products [48, 49].

1.4 Food security

The concept of food security, encapsulating food safety and sustainability is a versatile approach to sustainably ensure that everyone, now and in the generations to come, have physical and financial access to sufficient, safe, and nutritious food to meet their dietary needs and food preferences to live an active and healthy life (SDG). Food security goes beyond global phenomenon, but it must also be realized at individual household, national, and regional scale [50]. Keeping in mind that food security is intricately interwoven with food safety, and sustainability, a balance between food safety and sustainability must therefore be established so that at the end of the day, the United Nation sustainable development goal of poverty and hunger eradication, access to clean water, reducing terrestrial ecology footprint, addressing global warming phenomenon, sustainable production of food, and sustainable aquatic and terrestrial life, are fully realized [51]. According to the UNICEF, 22,000 children die every day because of malnutrition, there are around 750 million individuals who do not have access to clean drinking water, and more than 2 billion individuals are deficient in micronutrients. The problem of food security has potential to be further exacerbated due to accumulated stress on terrestrial natural resources and climate change [52, 53]. The menace of food insecurity, poverty, disease, and malnutrition that's has plague human development can be view closely by region (Figure 9). Although less pronounced in central Asia and Europe, however, low birthweight, childhood overweight, and adult obesity are more conspicuous and alarming withing these subregion [54].

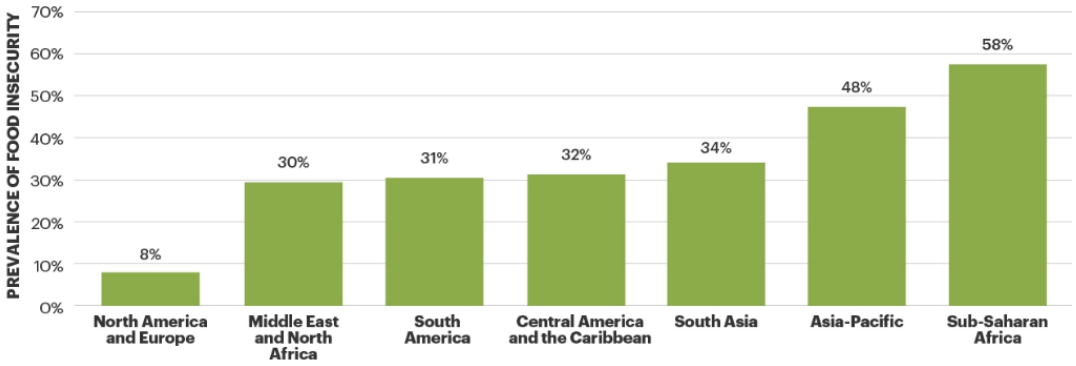

Figure 9: Per capital food insecurity by region as of 2018 [55].

Several measures have been put in place to address the issues of food security. Nevertheless, non-developed countries in South Asia and Sub-Saharan Africa are still far behind in attaining the feat of food security (Figure 9), ending hunger and poverty. Furthermore, the food insecurity place heavy burden on non-communicable disease, because of poor and insufficient nutrition, which leads to increased vulnerability and decreased adaptability to non-communicable diseases (NCDs) [56].

1.5 Non-communicable disease

In addition to 2 billion individual deficient in micronutrient and 22,000 children death due to poor nutrition, non-communicable diseases (NCDs) are responsible for 70% (39.5 million) of the global death (Figure 10) and this is expected to rise to 52 million in the year 2030 [57, 58]. Evidently, cancer, diabetes, chronic respiratory disease, cardiovascular diseases contributed immensely to the NCDs related death [59]. Triple burden of diseases which consisted of non-communicable diseases (NCDs), communicable, maternal, neonatal and nutritional diseases (CMNNDs) and injuries (Figure 10) have predominantly ravaged the economic and social development of developing and under developed countries, in these region, 48% (30.7 million) of people die before the age of 70 years, juxtaposing 26% in developed countries [59]. Insufficient nutrition resulting in less active individuals is the bedrock causes of obesity, type 2 diabetes mellitus and cardiovascular disease. Furthermore, Poverty, environmental degradation, and high population in these regions contributed immensely to food insecurities, which therefore worsen the situation of NCDs due to inadequate or unavailability of proper nutrient to manage it. Out of 169 target of sustainable development goal, reducing premature deaths caused by non-communicable disease by one third, and eradicating malnutrition have received much attention [60]. It is estimated that the rate of death for obesity based NCDs will rise to 46% by the year 2030 [61]. Non-communicable disease caused by malnutrition are the main cause of death and are more pronounced in the low- and middle-income countries. It is also important to realized that mass migration, globalization, increasing sedentary lifestyle, urbanization have enormous contribution to prevalence of non-communicable disease due to excessive calory intake, sedentary lifestyle, and tobacco consumption that eventually led to overweight, then obesity, high blood cholesterol, increase in blood pressure and eventually resulting in death [62]. Non communicable disease was included as part of sustainable development goals to foster development in cardiovascular disease eradication. As a result, a new approach is required in addition to previously established measure to effectively treat the non-communicable related diseases [63].

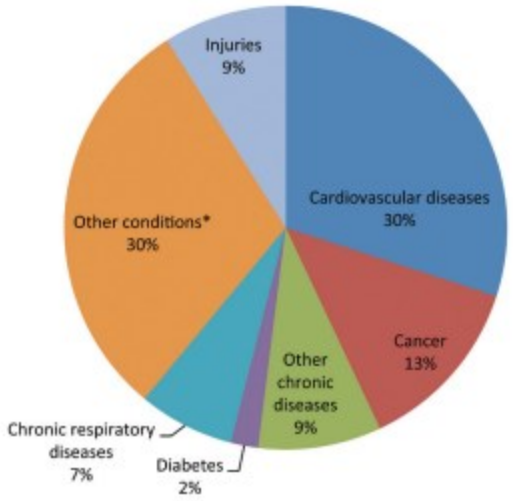

Figure 10: Contribution of non-communicable disease to global death, and triple burden of diseases (communicable, non-communicable, and injuries). Other condition refers to communicable diseases, maternal and perinatal conditions, and nutritional deficiencies.

1.6 Reactive oxygen species and oxidative stress

Oxygen plays a central/inevitable role in aerobic metabolism, in redox reaction that leads to generation of ATP by serving as electron acceptor in the cellular oxidation-reduction reaction. During cellular redox reaction, electron can sometimes transfer unpaired in an uncouple reaction which in turn leads to the production of free radical (Figure 11). These radicals are very unstable and reactive can quickly react with molecules within the system (Figure 13). Three different elements that can form the reaction center of free radical are nitrogen, oxygen, and sulfur. Example of the radical's form in reactive oxygen species include superoxide ($O_2 \cdot -$), hydroxyl ($HO\cdot$), peroxyl ($ROO\cdot$), alkoxyl ($RO\cdot$) and nitric oxide ($NO\cdot$) (Table 1**:** Various reactive oxygen species (ROS), reactive nitrogen species (RNS), and non-free radical species present in the cell [64]).

$$O_2 \xrightarrow{e^-} O_2^{-} \xrightarrow{e^-} O_2^{2-} \xrightarrow{e^-} OH\cdot \xrightarrow{e^-} H_2O.$$

Figure 11: Sequential abstraction of electron (e^-) from oxygen atom to producing free radical intermediate until finally reduced to water molecule [64]

The harm free radical produce is almost certainly unavoidable but can be salvaged by the repair mechanisms. Also present in the living system are non-radical ROS like singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). Scavenging the ROS is critical for cell homeostasis, which living creatures achieve through an antioxidant defense mechanism that allows them to maintain a balance between oxidative stress and antioxidant defense. An imbalance between ROS and antioxidant defenses causes oxidative stress. This oxidative stress disrupts several cellular functions and leads to several pathological conditions in which ROS overwhelm the organism's antioxidative defenses, causing oxidative modification of biological

macromolecules such as protein, carbohydrate, lipids and nucleic acids, tissue injury, and accelerated cellular death, which is at the root of many NCDs diseases. ROS and RNS are very unstable and have lower half-life and can quickly damage the tissues (Figure 12) [64]. At low level, ROS and RNS have been identified to participate in the regulation of homeostasis and body physiology by modulating transcription factors, protein phosphorylation, and ion transfer involved in cell signaling. However, high level of ROS and RNS accumulate in the cell and disrupt the natural biochemical reactions [65].

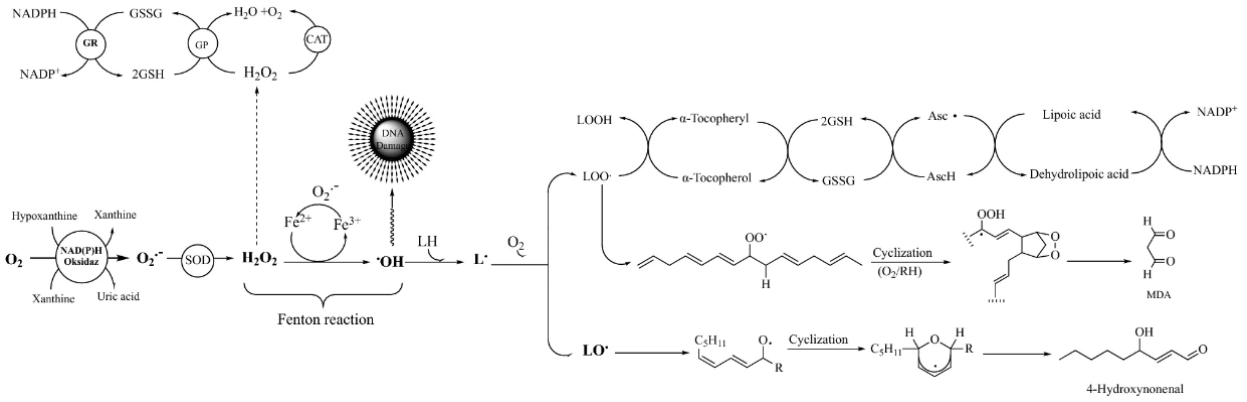

Figure 12: Reaction summary showing the formation of ROS and it effect on cellular metabolite *CAT* catalase enzyme, *SOD* superoxide dismutase enzyme, *GP* glutathione peroxidase, *GR* Glutathione reductase [64].

Table 1: Various reactive oxygen species (ROS), reactive nitrogen species (RNS), and non-free radical species present in the cell [64]

Reactive oxygen species		Non free-radical species	
Hydroxyl radical	HO·	Hydrogen peroxide	H_2O_2
Superoxide radical	O_2^-	Singlet oxygen	$^1O_2$
Hydroperoxyl radical	HOO·	Ozone	O_3
Lipid radical	L·	Lipid hydroperoxide	LOOH
Lipid peroxyl radical	LOO·	Hypochlorite	HOCl
Peroxyl radical	ROO·	Peroxynitrite	$ONOO^-$
Lipid alkoxyl radical	LO·	Dinitrogen trioxide	N_2O_3
Nitrogen dioxide radical	NO_2·	Nitrous acid	HNO_2
Nitric oxide radical	NO·	Nitryl chloride	NO_2Cl
Nitrosyl cation	NO^+	Nitroxyl anion	NO^-
Thiyl radical	RS·	Peroxynitrous acid	ONOOH
Protein radical	P·	Nitrous oxide	N_2O

a. $OH^{\cdot} + RS^{-} \rightarrow OH^{-} + RS^{\cdot}$
b. $CCl_3^{\cdot} + RH \rightarrow CHCl_3 + R^{\cdot}$
c. $CCl_3^{\cdot} + CCl_3^{\cdot} \rightarrow CH_2Cl_6$
d. $CCl_3^{\cdot} + CH_2 = CH_2 \rightarrow CH_2(CCl_3) - CH_2$
e. $CH_3CH_2^{\cdot} + CH_3CH_2^{\cdot} \rightarrow CH_2 = CH_2 + CH_3 - CH_3$

Figure 13: Different reaction mechanism of free radicals with surrounding chemical compounds, (a) electron donation, electron acceptance, reducing radicals, and oxidizing radicals, (b) hydrogen abstraction, (c) Self-annihilation reaction, (d) addition reaction, and (e) disproportionation reaction [64]

1.7 Marine amino acids and proximate composition

Many enzymes, hormones, neurotransmitters, nucleic acids, and other molecules required for life are made up of amino acids, which are the building blocks of protein. There are three types of amino acids which are: essential, nonessential, and conditional essential amino acids. Amino acids are critical regulators of gene expression and protein phosphorylation cascades, as well as nutrient transport and metabolism in animal cells, and innate and cell-mediated immunological responses. Lysine for example is essential for normal growth, and a lack of it causes immunodeficiency [66]. Arginine is necessary for cell division, wound healing, immunological function, blood coagulation, and blood pressure regulation, and Glycine aids in metabolic regulation, anti-oxidant activity, protein synthesis, and wound healing. [67]. Data from marine fish research has shown considerably high in essential amino acid, for example there is high content of lysin in marine fish as compared to that found in cereals or freshwater species [68]. As a result, a greater understanding of fish nutritional contents, which are believed to be strongly linked to fish species, could aid in the understanding of diversity among marine and freshwater fish species. Proximate composition of mesopelagic fish interestingly varies with oceans depth and regional productivity. Water content is higher in species living at the depth greater than 200m than those found on the surface. Therefore, with increasing depth of occurrence, there is a predictable drop in protein (percent wet wt) and lipid (percent wet wt). Species who live in oligotrophic locations have higher protein levels (percent wet wt) and lower lipid levels (percent wet wt) than those who live in high-productivity areas. Therefore, mesopelagic region is a great place to look at chemical composition trends at different latitudes [69, 70].

1.8 Marine bioactive peptides

Marine fish proteins upon hydrolysis with exogenous or endogenous enzyme release peptide sequences of different molecular weight. Some of these peptide's sequences have been shown to have a promising role in modulating enzyme or biomarkers of metabolic pathways of diseases, and by binding the cellular membrane. They are so called bioactive; several bioactivities have been documented in both marine [43], and diary raw materials. The bioactivities of the release peptides work in a structure-function manners and depends on the nature of the amino acid that constituted the peptides [71]. Depending on the purpose of hydrolysis, temperature, pH, choice of enzyme and

the amount can be carefully selected for in such a way to optimize the bioactivities of the hydrolysates [70, 72]. Cardiovascular disease and type-2 diabetes mellitus have immense contribution to non-communicable disease associated death in Europe and globally [73]. Example pathway's modification in NCDs by bioactive peptides are when glucagon-like peptide (GLP-1) are being release upon stimulation, inhibition of renin-angiotensin system (ACE). inhibition of dipeptidyl peptidase-IV (DPP-IV) by stimulating incretin system, anticoagulant capacities, and appetite suppression by stimulating the secretion of intestinal cholecystokinin. In addition, bioactive peptide can also act as free radical scavenger in a manner that reduce oxidative stress on membrane, and therefore preventing cascade of reaction leading to cardiovascular diseases, diabetes, ischemia, cancer, and neurodegenerative disease (Figure 14) [74]. The marine peptide is processed into functional food product and nutraceuticals to manage NCDs symptoms. Low molecular weight peptide (di- and tripeptides) have been identified to have increased bioactivities, and high molecular weight peptide show improve functional properties [75, 76].

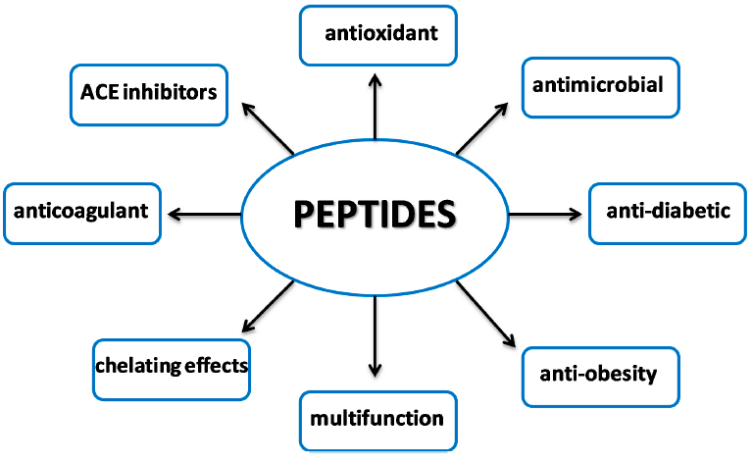

Figure 14: Bioactivity modulatory effect of marine peptides [20].

1.9 Measurement of antioxidant capacity

Selecting an appropriate assay based on the chemical(s) of interest is crucial when investigating antioxidant activity in food. Various chemical processes can be used to determine the antioxidant activity of peptides *in vitro*. Using different assay methods can help in proper establishment of antioxidant activity of natural product. Methods for determining antioxidant capacity can be categorized based on whether they measure hydrogen atom transfer (HAT) or electron transfer (ET) [77]. The oxygen radical absorbance capacity (ORAC), hydroxyl radical, alkyl radical, and peroxide radical scavenging activity assays are all used to measure proton-donating ability. The ET based approach in determining antioxidant capacities are the Trolox equivalent antioxidant capacity (TEAC), 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation (ABTS•+), FRAP (ferric reducing antioxidant power), and DPPH• (2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl radical) assays [78]. The presence of specific amino acids in bioactive peptides, particularly histidine residues, has been ascribed to their antioxidant effectiveness. The imidazole ring's chelating and radical-trapping characteristics are thought to be responsible for this. In

addition, the presence of hydrophobic amino acid residues in peptides has been linked to increased accessibility to hydrophobic targets [79, 80].

The ORAC method is used to map the oxidative breakdown of fluorescent molecule, like β-cyclodextrin or fluorescein with free radical generator in the medium, such as the such as azo-initiator compounds. The Azo initiators generate ROO· when the temperature is high, and this process destroy the fluorescent molecule and cause the fluorescence to fade in a progressive manner that generate nonfluorescent compounds [81], as the oxidative degeneration reaction proceed (Figure 15) [82]. The protection confers on the fluorescent molecule by an antioxidant compound from undergoing oxidative degeneration can be measured fluorometrically and the antioxidant capacity which quantify the amount of product formed in time frame is determined by ORAC equipment.

$$R - N = N = R \xrightarrow{O_2} N_2 + 2ROO^{\bullet},$$

$$ROO^{\bullet} + Probe_{(Fluorescent)} \rightarrow ROOH + Probe_{(Non-fluorescent)},$$

$$AH + ROO\cdot \rightarrow ROOH + A\cdot,$$

$$A\cdot + ROO\cdot \rightarrow ROO - A.$$

Figure 15: Reaction mechanism of ORAC [64].

The ABTS is a HAT and ET method based, which involve the oxidation of neutral solution to a radical cation, ABTS·+ with deep color (Figure 16). The antioxidant is measure is measured by testing the proposed natural compound to reduce the intensity of the radical cation solution. ABTS can be used over a wide range of pH which is useful to study how pH modulates the mechanism of antioxidation. Furthermore, ABTS is stable is in both organic and aqueous solution, which broaden it usage [83]. In general, this spectrophotometric method based on the ABTS radical cation has been used to measure the antioxidant capacity of natural substance [64].

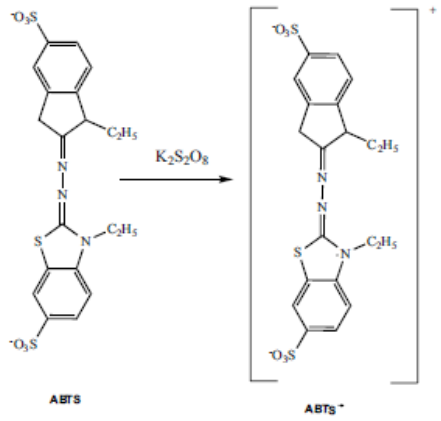

Figure 16: Reaction mechanism of ABTS [64]

FRAP is an electron-based (ET) which use spectrophotometer at 593 nm to map the reduction rate of ferric ions (Fe^{3+})–ligand complex (ferric 2,4,6-tripyridyl-*s*-triazine complex $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$ to the intensely blue-colored ferrous ions (Fe^{2+}) complex $[Fe^{2+}\text{-}(TPTZ)_2]^{2+}$ in the presence of antioxidant and lower pH (3.6) to sustain the iron solubility (Figure 17) [64]. Although there is a debate which tried to disprove the relationship between iron reduction potential and the radical scavenging process of proton transfer of most antioxidant. However, reducing power represents a compound's ability to modify redox tone in plasma and tissues, and oxidation or reduction of radicals to ions still stops radical chains [84].

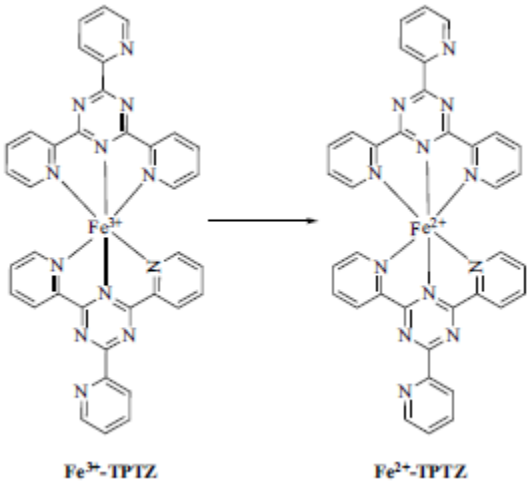

Figure 17: Reaction mechanism of FRAP [64]

The DPPH is a stable radical of nitrogen with maximum absorbance of 517 nm, in which the spare of electron on nitrogen is delocalized throughout the entire molecule giving rise to deep violet color and prevent the molecule from undergoing dimerization. In the presence of antioxidant molecules that donate H atom to the DPPH radical, the violet color is loss, absorbance of the DPPH is reduced (Figure 18) [82]. The sensitivity of the DPPH can be affected by amount and type of solvent used, and the presence of hydrogen and metal ions in the freshly prepared DPPH [85].

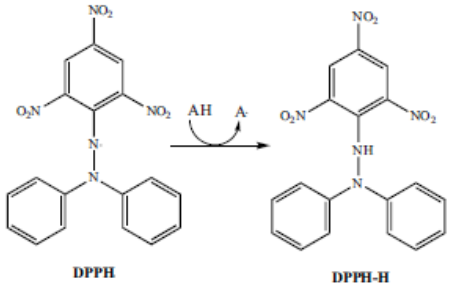

Figure 18: Overview of DPPH reaction [64].

1.10 Angiotensin-converting enzyme (ACE) inhibitory effect

Blood pressure can be modulated by changing the volume of blood flow, hormone secretion, endothelial cell and renin-angiotensin-aldosterone secreting nitric oxide. In the renin-angiotensin-aldosterone system (RAS), ACE is an enzyme that convert angiotensin I to II and this result in constriction of the blood vesicles and increase in blood pressure [86]. ACE can also modulate this system by degrading bradykinin, a vasodilator then leading to lowering in blood pressure. Peptides that have the potential to bind serum binding protein (SBP), inhibit ACE or stimulate production of nitric oxide (NO) in the endothelial cells are very promising in treating hypertension (Figure 19). These peptides are mostly short sequence, have an alpha helix (α 1-3) amino structure in terminal ends of its structure. *In silico* analysis of 4-10 and 2-3 bioactive peptide has shown that, from the C terminal ends, and presence of Tyr and Cys leads to more potent ACE activities, this is followed by Trp, Met and His, then Leu, Ile, Val, and Met, and Trp in the last position [87]. Although system that regulate blood system goes beyond modulation of ACE inhibitory activities, however bioactive peptide derive form fish protein hydrolysate has can also significantly modulate this process [88].

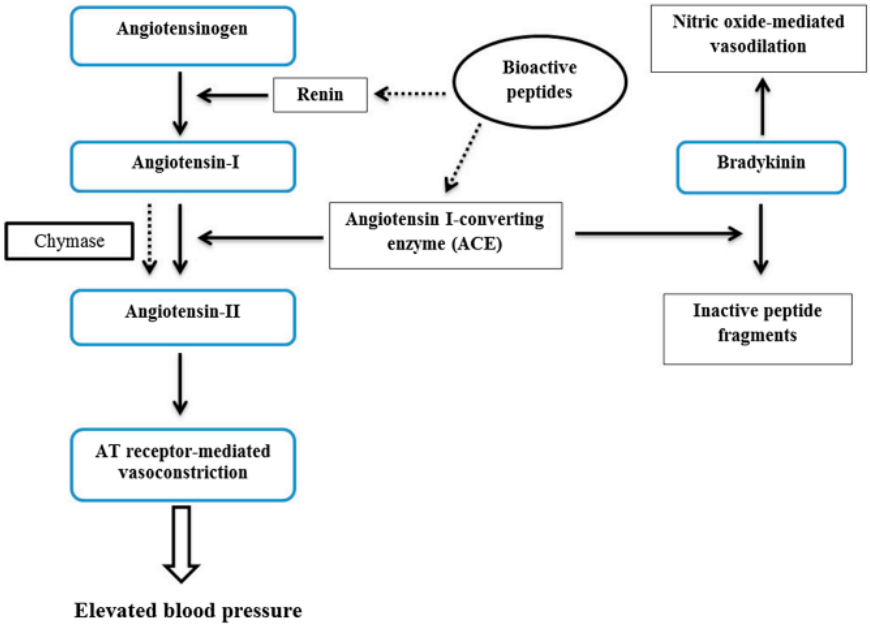

Figure 19: Regulation of blood pressure through renin-angiotensin-aldosterone system (RAS) pathway [89]

1.11 Molecular peptide sequencing

Membrane filtration and chromatographic techniques are commonly employed to separate and purify protein hydrolysate. Aside being economical and sustainable, the membrane filtration is particularly important because it is effective in separating peptides based on molecular weight [90]. Separation based on molecular weight (kDa) is crucial as studies have revealed the relationship between bioactivity of fish peptide and the molecular weight (Figure 20). The marine peptides of low molecular weight (0-3kDa) and 3-20 amino acids in length have innately higher bioactivities than the larger peptides of (3-10kDa) [91]. Different chromatography techniques have been developed for protein separation based on their mobile or stationary phase affinity. The most relevant technique in this regard is the high-performance liquid chromatography (HPLC) which can efficiently separate small peptide fraction and generally a high-throughput techniques compare to other chromatographic techniques. This technique, due to it high resolution, is evidently used in the final purification of bioactive peptides [92]. Example of some relevant antioxidative and anti-hypertensive peptides were listed (Table 2).

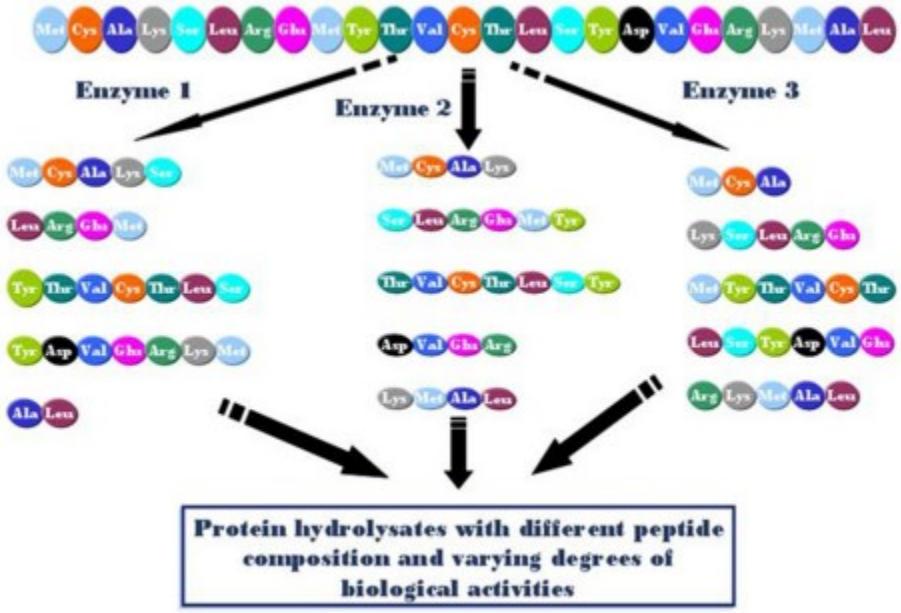

Figure 20: Generation of protein hydrolysate by action of different enzyme which yield varying peptide length and size [18].

Table 2: Example of Antioxidative and ACE inhibitory peptides [18] [93]

Effects	Amino Acid Sequence	Protein Substrate	Reference
Antioxidant Activity	Asn-His-Arg-Tyr-Asp-Arg	Skin protein of horse mackerel	[94]
	Gly-Asn-Arg-Gly-Phe-Ala-Cys-Arg-His-Ala	Skin protein of croaker	[94]
	Leu-Asp-Lys	Sphyrna lewini muscle	[95]
	Ala-Met-Thr-Gly-Leu-Glu-Ala	Black pomfret viscera protein	[96]
	Leu-His-Tyr	Sardinella aurita	[90]
	Trp-Glu-Gly-Pro-Lys	Bluefin leatherjacket head	[97]
	Gly-Pro-Pro		
	Gly-Val-Pro-Leu-Thr		
ACE Inhibiting peptides	Val-Leu-Trp, Val-Phe-Tyr, Leu-Ala-Phe	Salmon protein	[98]
	Gly-Pro-Leu-Gly-Leu-Leu-Gly-Phe-Leu-Gly-Pro-Leu-Gly-Leu-Ser	Hydrolysate from squid gelatin	[99]
	Val-Tyr-Ala-Pro-, Val-Ile-Ile-Phe	Cuttlefish muscle	[100]
	Phe-Leu	Salmon muscle	[101]
	Cys-Phe, Glu-Tyr-, Met-Phe, Phe-Glu, Cys-Phe, Glu-Tyr, Phe-Glu	Shark meat	[102]

1.12 Protein yield

The production of protein hydrolysate with commercial enzyme is frequently used, nevertheless expensive [103]. On the other hands, autolytic approach which use the innate enzyme present in the natural product is more economical and sustainable, but ineffectual in generating hydrolysate with some desired physical and functional characteristic, and thus limited usage in preparation of hydrolysate [37]. Furthermore, change in environmental factor, season, age, gender modulate the nature and amount of endogenous enzyme present in the fish tissue, and this ultimately affect the yield of the protein hydrolysate, meaning autolysis is not stable [37]. The rate of breaking down of fish tissue with endogenous enzymes is slow compared to the enzymatic hydrolysis or bacterial fermentation which progress rapidly, and yield more protein per raw materials, thus making it

more applicable in animal feed production [37]. Moreover, enzymatic based hydrolysis can be tuned favorably to meet the desire functional properties of the fish product.

1.13 Application of marine fish derived peptides

Bioactive peptide extracted from marine resources have been used in a wide range of industrial and commercial application. *In vitro* studies of many such peptides have indicated positive health effect and have been modified into capsules for pharmaceuticals as in in drug delivery, with desired peptide length, this capsule on getting to the GIT can then be released to bind the target site of the disease [104]. Application of marine peptides in the cosmetic industry has also received large attention, incorporation of antioxidant peptides in cosmetics may potentially help in designing photo-protective and antiaging cosmeceuticals [43]. Since it is relatively easy to control the techno-functional properties like foaming and emulsion during the enzymatic hydrolysis of the protein hydrolysate, this will be of great importance in the food industry serving as food stabilizer. Furthermore, marine peptide has potential to replace the conventional artificial or chemical preservative due to their high tendency to prolong the shelf life of food [105]. Additionally, the widely known antioxidant attribute of marine peptide can be applied in preserving fish product by acting as antioxidant and cryoprotectants, and therefore preventing essential marine oil from undergoing lipid oxidation [106]. Also, marine peptide can be used to retain the gel forming ability in seafood product, this is more suitable as sugar molecule already in used give characteristics sweetness under frozen condition to the preserved product [107].

2 Materials and methods

General overview showing step by step process from handling of raw materials to the generation of the protein hydrolysate. The processing of protein hydrolysate of batch A and batch B were carried out in October 2021 and January 2022 respectively (Figure 21).

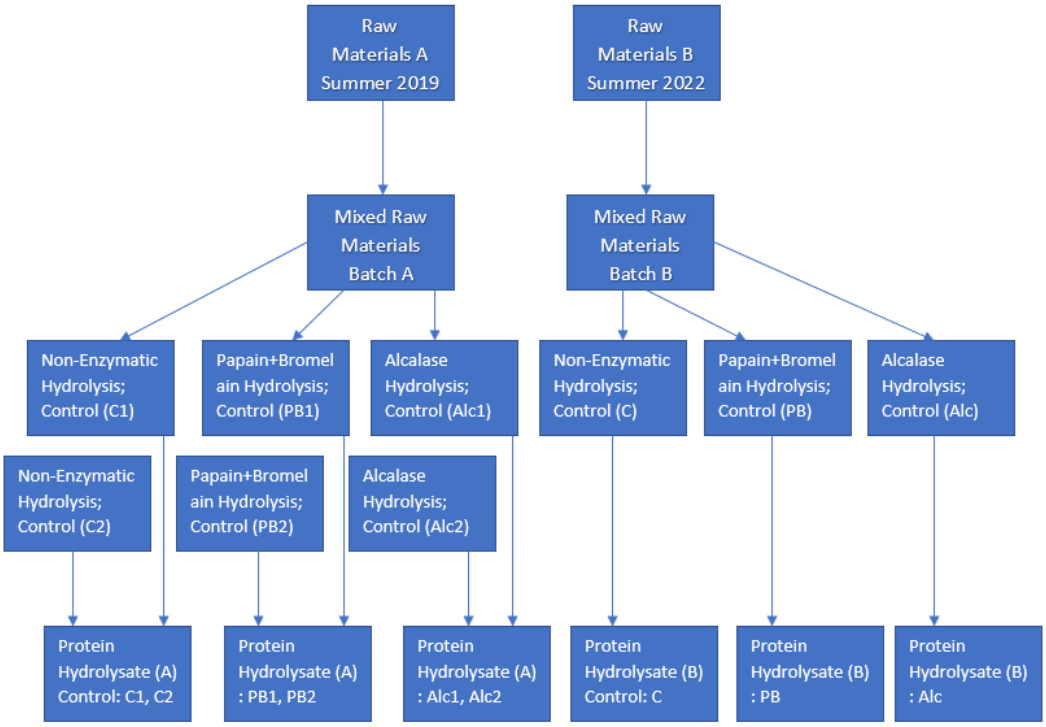

Figure 21: Process flow and overview from handling of the raw materials to the production of protein hydrolysate. Sample A, krill and pearlside; sample B, pearlside. Control (C), raw materials hydrolyzed without enzyme; papain+bromelain (PB), raw materials hydrolyzed with these two enzymes; alcalase (Alc), raw materials hydrolyzed with enzyme alcalase. Sample A was analyzed in duplicate bioreactior C1, C2, PB1, PB2, Alc1, Alc2. Sample B was analyzed in a single bioreactor C, PB, Alc.

2.1 Procurement of raw materials

The mesopelagic fish utilized in this thesis are Pearlside *(Maurolicus muelleri)* and Northern krill *(Meganyctiphanes norvegica)*. The samples were harvested at two different time points. One batch (A) was caught with an adaptive pelagic trawl by Liegruppen AS, at 175 m depth, around N59° 30.', E 03° 21. in Norskerenna, summer 2019. The other batch (B) was caught in summer 2022 around N58° 51', E003° 39' at 190 m depth. Batch A containing two mesopelagic fish species (Krill and Pearlside), arrived from Tromsø on 26th of August 2021. Batch B containing only

Pearlside, arrived from Tromsø 17th January 2022. Both batches were stored at -80ºC freezer (Figure 22). For each batch, the frozen sample was allowed to thaw for 30 minutes and minced using a meat grinder (Bosch, Germany). The minced sample was weighed, divided into zip-lock bags, and stored at -80℃ freezer for further analysis.

Figure 22: Frozen mixed mesopelagic sample of Batch A containing Pearlside *(Maurolicus muelleri),* and Northern krill *(Meganyctiphanes norvegica)* analyzed as a pooled sample and Batch B containing only Pearlside *(Maurolicus muelleri).*

2.2 Hydrolysis (Enzymatic and non-enzymatic)

Non-enzymatic and enzymatic hydrolysis of mesopelagic fish were carried out in a bioreactor, containing two main compartments; the temperature control system and the main chamber where the hydrolysis reaction proceeds. The raw (300 g) was thawed at room temperature and mixed with water (300 g) before subjected to hydrolysis in a bioreactor (Figure 23). Stable temperature was reached (49±1ºC) before starting the hydrolysis by adding alcalase (0,1% of wet weight raw material) or papain and bromelain (0,05% of wet weight raw material of both enzymes), no enzyme was added for control. The pH was measured (6.5-7.5). The hydrolysis was performed for 1 hour with stirring (400 rpm).

Figure 23: Real-time bioreactor set up showing the temperature controller (A) where deionized water is heated up till 50ºC and reactor chamber (B) where the hydrolysis proceeds with monitored temperature and pH.

At the end of hydrolysis, the sample was heated to 100°C and kept for 10 minutes to inactivate the enzymes. Upon enzyme inactivation, the samples were cooled down to room temperature before centrifugation at 5000rpm for 30 minutes to separate the phases. The protein hydrolysate was separated out as the clear liquid middle phase between the sludge and lipid phase, and the sediment/debris (Figure 24). Following the centrifugation, the samples were frozen at -40ºC for later separation.

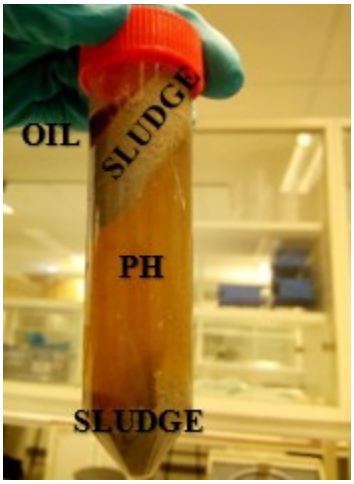

Figure 24: Illustration of the centrifuged protein hydrolysate sample showing the middle clear brownish colored protein hydrolysate (PH), the raw fish meat partly sediment at the top and bottom of the tube, while the lipid was deflected to the upper top left side of the tube.

The frozen samples were cut, separating the protein portion (middle part) from the oil sludge (top part) and sludge (bottom part) (Figure 24). The separated protein phase was sieved through a glass wool, frozen at -40ºC and later freeze-dried for one week. The dried samples were grounded into mesopelagic protein powder using mortar and pestle, transferred into air-tight zip-lock bags and stored at -80ºC freezer (Figure 25).

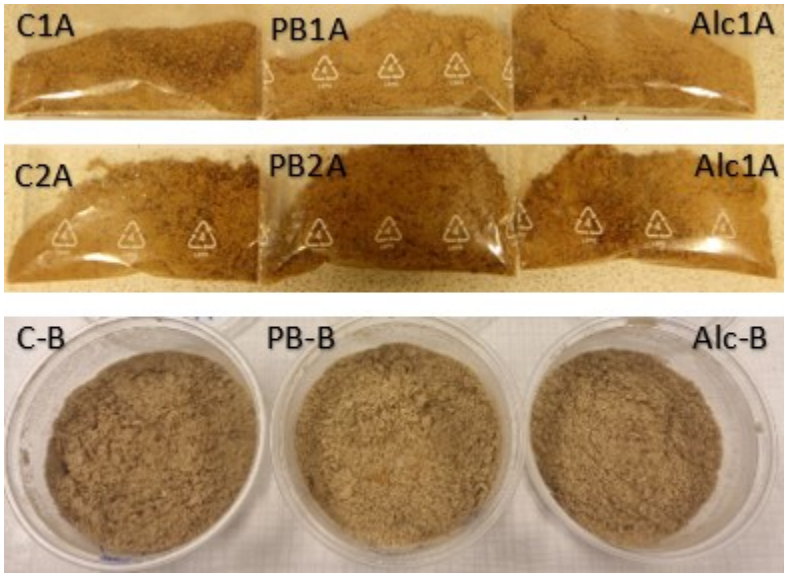

Figure 25: Mesopelagic fish protein powder after the freeze-drying process. Control (C), Papain + Bromelain (PB), Alcalase (Alc), Batch A containing pearlsides and krill (A) and Batch B containing pearlside (B).

2.3 Lipid extraction

The lipid content determination was carried out by two methods, the macro, and the micro methods. For the macro method, 10 grams of raw materials were weighed into the centrifuge tube and placed on the ice prior the analysis (Bligh and Dyer method). For each sample, 10mL of distilled water, 20mL of chloroform, and 40mL of cold methanol was added respectively. The solvents were added in the specific manner of polar to non-polar to effectively extract the lipid and dissolved each phase in the mesopelagic fish samples [108]. Further the samples were homogenized using Ultra Turrax for 2 minutes at the speed of 17000rpm. 20mL of chloroform was added to the sample, and again was homogenized for 30 seconds at the speed of 17000rpm. After the homogenization, the addition of 20mL of distilled water to the mixture which were then homogenized for additional 30 seconds at the rotational speed of 17000rpm. The sample was centrifuged at the centrifugal speed of 4800rpm for 10 minutes. Centrifugation partitioned the mixture into three layers: the lipid-containing chloroform phase (middle part), water and methanol

phase (top part), and the fish meat phase (bottom part). 2mL of the chloroform phase was added to the pre-weighed Kimax bottles and evaporated under the stream of N2 gas at 60°C for period of 1 hour in the evaporation unit. The evaporated samples were transferred to the desiccator and the lipid content was determined. The micro method which was applicable to protein hydrolysate was almost like the macro methods except that, due to limited quantity of samples, 1 gram of the protein hydrolysate was weighed instead of 10 gram in the macro method and all the volume in the macro method were divided by value of 10. The analysis was performed in triplicate for the raw materials and duplicate for the protein hydrolysate

The total lipid contents were calculated to depict quantity of lipid sample extracted per 100g sample in the following equation.

$$\%\ \text{Total Lipid} = \frac{\text{lipid mass after evaporation (g)}\ .\ \text{total volume CF added (mL)}}{\text{chloroform extract (mL)}\ .\ \text{sample weight (g)}}\ .100 \quad (1)$$

$$\text{Weight of lipid samples (W)} = \frac{(\text{C} - \text{A})}{\text{Vchlo}} \quad (2)$$

$$\text{Weight of lipid after evaporation (D)} = \text{B} - \text{A} \quad (3)$$

$$\%\ \text{Total lipids} = \frac{(\text{C} - \text{A}).\ \text{Total volume of cloroform added}}{\text{Weight of raw materials}} \quad (4)$$

Where,

CF = Chloroform

C = Weigh of Kimax tube + lipid sample after evaporation A = Weight of empty tube

Vchlo is the volume of chloroform extract for evaporation (2mL)

D = Weigh of sample after evaporation (lipid in the kimax tube)

B = Weight of sample + kimax tube

2.4 Water content

The water content was performed as stated in the official method of analysis (AOAC, 2005). 2 grams of raw sample and 0.2 gram of protein hydrolysate was added into a pre-weighed porcelain crucibles and placed into a drying oven at 105°C for 24 hrs. Furthermore, the samples were transferred to a desiccator, cooled down to room temperature before weighing. The analysis was performed in triplicate for the raw materials and duplicate for the protein hydrolysates. The water content was calculated according to equation 5.

$$\% \text{ Water Content} = \frac{WWS - WSAD}{WWS} . 100 \quad (5)$$

Where WWS = wet weight of samples (g),

WSAD = weight of the samples (g) after drying.

2.5 Ash content

The dried mesopelagic fish samples were placed in the muffle furnace and were allowed to be burnt at 550℃ overnight according to method described in the official method of analysis (AOAC, 2005). The burnt sample were transferred into a porcelain crucible and were allowed to cool down to room temperature before weighing. The percentage ash content was determined according to the equation 6.

$$\% \text{ Ash content} = \frac{WSAB}{WWS} . 100 \quad (6)$$

Where WSAB = weight of the sample (g) after burning,

WWS = wet weight of the sample (g)

2.6 Protein content

Protein content was determined according Kjeldahl method [109]. Depending on sample analysed, 1. 5 grams of raw the mesopelagic fish and 0.5 grams of protein hydrolysate were weighed with whatman Kjeldahl analysis weighing paper (grade B-2) and placed in the 300mL sample tube. Two catalytic tablet of potassium sulphate (K2SO4) and15mL of sulfuric acid (conc.98%) were added to each tube for the digestion of the organic matter. Tubes with only weighing paper served as blank. Glycine (0.2 grams) served as internal. The digestion process was started immediately based on the parameters outline in the (Table 3) and was allowed to procced until the liquid present in the sample tube turned clear and blue green signifying the completion of the digestion. The sample were then allowed to cool down at the end of the digestion, equation **7** shows the digestion reaction. The sample rack was transferred to the KjelMaster K-375 with KjelSampler K-376 for distillation and titration. In the automated process, the percentage of nitrogen was calculated and sample specific protein factor for fish (6.25) was multiplied with the percentage of the nitrogen to obtain the protein content.

Table 3: Temperature profile for digestion with the *K-449*

Step	Temperature (°C)	Time (min)
1	280	0
2	320	20
3	420	90
Cooling	-	35

$$(C_nH_nN_nO_n) + H_2SO_4 \longrightarrow CO_2 + SO_2 + H_2O + NH_4^+ \quad (7)$$

2.7 Total amino acid

The procedure for the total amino acid analysis was conducted as described by [110]. The determination of total amino acid was started by hydrolysis of mesopelagic fish raw materials in 1mL of 6M HCL. Approximately 0.05 grams of raw materials and protein hydrolysate were weighed in the flat-bottomed glass tube and put in oven for 22 hours at 105ºC for the complete hydrolysis of the connective tissues. The hydrolyzed sample were allowed to cool down to room temperature and the constituents were flushed with deionized water into 10ml beaker. The hydrolyzed solution was titrated to a neutral pH of 7.0 with HCL and NaOH. The neutral solution of the sample was filtered by suction with the aid of Whatman glass microfibers filter GF/C, and the filtered sample was transferred in to the 10ml flask and filled up to the mark with deionized water. This was followed by additional filtration through 0.22µm filter using syringe. For the raw materials, the sample was diluted 1:500 with deionized water while the protein hydrolysate was diluted 1:1000, this is to ensure that the HPLC efficiently read all the amino acid. 0.205 ml of the appropriately diluted solution was transferred into the tube for the HPLC analysis.

2.8 Free amino acid

The procedure was followed as described by [111]. The determination of free amino acid started with the preparation of the extract, ~0.1 gram of samples was weighed and dissolved in the deionized water, then subjected centrifugation at 5000rpm for 15 minutes. 1 mL supernatant was transferred into Eppendorf tube, 0.25mL 10% sulfosalicylic acid was added, shake, and left in the cold room at 4ºC for 30 minutes. The analysis was performed in triplicate for the raw materials and duplicate for the protein hydrolysates. The mixture was centrifuge at higher centrifugal speed of 10,000rpm for 10 minutes to ensure all the precipitate settled at the bottom of the tube. 0.2mL of the supernatant was taken out of each parallel and diluted with 4.8mL of deionized water in 1:100 dilution, and the solutions were filtered with 0.2µm filter pore. 0.205ml was transferred into the tube after filtration for the HPLC analysis.

2.9 Degree of hydrolysis by formol titration

The formol titration was followed as described by [112]. Approximately 1.5 grams of both raw materials and mesopelagic fish protein hydrolysate was weighed, deionized water was added to this sample until the weight reach 50 grams and the resulting mixture was titrated with HCL and NaOH until the neutral pH of 7.0 was reached. Thereafter, 10ml of formaldehyde was added to the neutral mixture and allowed to stand for 5 minutes. The solution was titrated to pH 8.5 with NaOH. To know the rate of protein hydrolysis both in the samples, the concentration and volume of NaOH used was noted, and the nitrogen content calculated for the respective sample in (proximate composition) was used to calculate the degree of hydrolysis (%DH). This was done by first calculating the amount of free amino groups (D) and subsequently using this to calculate the %DH as shown in the equation 8 and 9 respectively.

$$\%\, Free\ amino\ groups = \frac{A * B * 14.007 * 100}{C * 1000} \quad (8)$$

A = NaOH used in ml
B = concentration of solution used for titration (0.1M NaOH)
C = weight of sample in grams
14,007 = molar mass of nitrogen (g/mol)

The degree of hydrolysis is then computed by dividing the % free amino groups by the amount of nitrogen present in the sample from Kjeldahl analysis.

$$\%\, DH = \frac{D}{E} * 100 \quad (9)$$

% DH = degree of hydrolysis in percentage.
D= % free amino groups
E = % Nitrogen (% protein divided by 6.25)

2.10 Yield

The yield was calculated based on the weight and protein content of hydrolysate powder compared to the weight and protein content of raw material. Approximately 300 grams of wet mesopelagic fish was weighed for each enzyme hydrolysis and the control. In the process of weighing, transferring sample into the bioreactor, and deactivation of enzymes in the microwaves, some samples were loss. Therefore, the final weight of mesopelagic fish was less than the weight recovered after the hydrolysis. Although this weight loss is insignificant compared to the whole sample weight, it is important to reiterate that the yield is not 100% accurate. Samples of batch A were running in two parallels/bioreactors while that of batch B were only ran with one parallel.

$$Yeild = \frac{Amount\ of\ Hydrolysate * Protein\ Concentration\ in\ Hydrolysate}{Amount\ of\ Raw\ Materials * Protein\ Concentration\ in\ Raw\ Materials} * 100$$

2.11 Bioactivity measurement

The concepts of bioactivity used in ferric reducing antioxidant assay (FRAP), oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was based on measuring the absorbance and kinetic degradation of known chemical reagent at varying concentration (µM) to accurately map the absorbance/ kinetic degradation of protein hydrolysate powder and then determine the concentration in µM [113]. A standard curve was made, and the reliability was established based on the coefficient of determination (R^2) (Figure 26), the closer this value gets to the unity, then the more reliable it will be. The slope of the standard curve and the intercept on the y-axis together with the measure absorbance was used to compute the concentration of unknown sample.

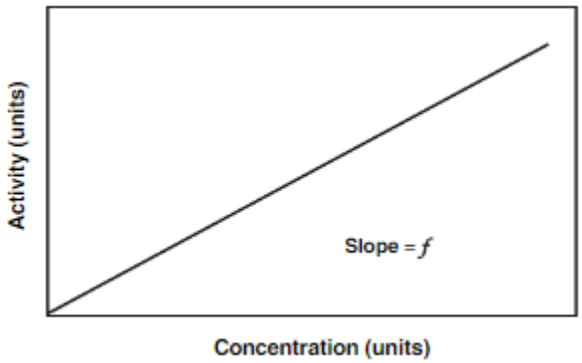

Figure 26: Graphical representation relating bioactivities to concentration [114].

2.11.1 FRAP (Ferric reducing antioxidant power assay)

The procedure followed for the FRAP assay was as described by [115]. 19mM $FeCl_3.6H_2O$ solution was prepared, 40mM concentrated HCl was made, and 10mM concentrated TPTZ solution was made by dilution with HCL. Acetate buffer was prepared by dissolving sodium acetate salt in glacial acetic acid and was fill to 500mL with water. Trolox was used as standard (Appendix A). FRAP working solution was made by mixing 5ml of Fe(III) solution, 5ml TPTZ solution, and 50ml of acetate buffer. The solution was incubated at 37ºC until use. The samples were appropriately diluted in water before centrifugation at 5000 rpm for 10 minutes. To the costar 96 flat bottom plate well, 10µL of sample or standard, 30µL dH_2O and 300µL FRAP solution were added, where the blank well consisted of 40µL dH_2O and 300µL FRAP solution, and reference was 340µL dH_2O. The mixture was incubated at 37ºC for 30 minutes, and the absorbance of the

sample and standard were read via the VMware workstation 12 player (Gen 5 2.00) at spectrophotometry of 593nm.

2.11.2 ABTS assay (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ABTS assay in this thesis followed the procedure described by [116-118]. The analysis started with the preparation of the ABTS* reaction solution. ABTS* was prepared by adding 7mM concentrated ABTS to 140mM concentrated $K_2S_2O_8$, and the reaction was allowed to proceed overnight while covering the bottles with aluminum foil to prevent the reaction mixture degradation. The reaction mixture was further diluted with 80% methanol until the absorbance was 0.75±0.05 at 734nm with water as a reference. Propyl gallate standard curve was used (Appendix A). 2ml of the ABTS• working solution was added to 200µl extract, standard and blank (80% methanol 0µM), this mixture is vortex well and incubated for 6 minutes at room temperature before the absorbance was read spectrophotometry at 734nm using deionized water as reference. The sample was appropriately diluted in 80% methanol, then centrifugation at 5000rpm for 10 minutes. After then, the extract was diluted 1:1000 which was achieved via trial and error until the absorbance fit into the standard curve.

2.11.3 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activities

The procedure followed for the DPPH were the ones described by [118, 119]. 10mM solution of propyl gallate was added to 80% methanol. 0.15mM DPPH solution was added to 96% ethanol and covered with aluminum foil which is left to stir overnight in the darkness at 4ºC. The propyl gallate was diluted serially to make the standard curve (Appendix A). Samples were weighed and suitably diluted in methanol; the final dilution suitable for the optimal absorbance was done by trying different dilution factor until the sample absorbance fall inside the standard curve. 1.5ml of diluted sample or standard was added to 1.5ml of DPPH and the mixture was vortex well. A blank tube was made that contain only 80% methanol; this mixture was allowed to incubate for 30 minutes in the drawer at room temperature. The absorbance was then read at 517nm using 96% ethanol as reference.

2.11.4 ORAC (Oxygen radical absorbance capacity)

The ORAC procedure followed in the experiment was modified from [120]. 75mM concentrated phosphate buffer (pH 7.4) was prepared, and 55nM concentrated Fluorescein (FL) was made from phosphate buffer solution. The Trolox was appropriately diluted serially with phosphate buffers and was used construct the standard curve (Appendix A). 153mM concentrated AAPH solution was prepared and kept on ice until use. The sample was appropriately diluted with water, then phosphate buffer. 25µL of the sample or the standard was micropipette into the black well assay plate, 25µL of phosphate buffer was added, and 150µL of fluorescein working solution was added with multi-channel pipette, the mixture then was incubated at 37ºC for 15 minutes. 50µL of AAPH

was quickly added with multi-channel pipette upon incubation and the sample was transferred to the plate reader where the kinetic fluorescence reading was started instantly. The kinetic fluorescence equipment and the cooling device have been put on while preparing the solution, this was done to allow the temperature sufficiently to reach 37ºC±5 and prevent the time lag between the start of the enzymatic reaction and for equipment to reach optimum temperature for the reaction.

2.12 Molecular weight distribution

The sample was prepared by dissolving finely grounded homogenous protein hydrolysate powder in water to a concentration of 10mg/ml, the sample was allowed to completely dissolve for one hour at room temperature with occasionally shaking. 1mL of the dissolved sample was filtered out with 0.22µm low protein binding microfilter into the HPLC vial. Then, 20µL of the filtered hydrolysate was injected on the chromatography column (Superdex 30 Increase 10/300 GL), while ensuring two successive runs per via. The molecular weight distribution segment of the peptides was assessed by peptide standard, and protein hydrolysate were filtered out through membrane filtration by membrane with distinct cut-offs. The standard used for the absolute quantification was made from hydrolyzed albumin (*in house - internal).* The chromatogram spot was integrated automatically by the HPLC software (HPLC-DAD from Agilent). The HPLC chromatogram were displayed in the (Appendix C).

2.13 Statistical analysis

Microsoft Excel 2016 was used for the data analysis and to compute the error. Results were presented as average ± standard deviation unless otherwise stated. IBM SPSS Statistics 26 with a significant level of 0.05 was used. The samples were assumed to be independent and normally distributed, with independent and random errors and equal variances. The error analysis for the antioxidant (FRAP, ABTS, DPPH, and ORAC) were included in the Appendix D.

3 Result and discussion

This section includes the result and discussion based on the experimental procedure outlined in the second chapter. Figures and tables that serve as basis of comparison can be found in the appendix B – C . For the antioxidant assays, the standard curve was included in the (Appendix A). During the experiment and the analysis, some abbreviation terms used include; batch A or raw material batch A (raw materials containing both krill and pearlside), batch B or raw material batch B (raw material containing only krill), protein hydrolysate A (protein hydrolysate recovered from raw material batch A) protein hydrolysate B (protein hydrolysate recovered from raw material batch B), control (raw materials hydrolyzed without adding enzyme), papain+bromelain (raw materials hydrolyzed with these two enzyme), and alcalase (raw materials hydrolyzed with enzyme alcalase).

3.1 Proximate composition

The proximate composition of the two batches of mesopelagic fish raw material were first analyzed and compared (Table 4). Water makes the highest contribution, with raw material batch A having a value of 70% and the raw material batch B having 72%. The protein content did not differ and was approximately 15 %, while the lipid content had a close value on average 11%. The ash content is the lowest with the value of 2.83 in raw material A and 0.36 in raw material B. The higher ash content of raw material A (krill and pearlside) can be associated with the presence of exoskeletal structure in krill which are made up of minerals [121], and thus confer high ash content as compared to the raw material B containing only pearlside.

Table 4: Proximate composition of raw materials of mesopelagic batches containing pearlside (batch A) and pearlside and krill (batch B). The results are presented as average (n=3) +/- stdev.

Samples	*Water Content (%)*	*Ash Content (%)*	*Lipid (%)*	*Protein (%)*
Raw Material batch A	70.61±0.41	2.83±0.07	12.03±0.82	14.75±0.23
Raw Material batch B	72.50±0.37	0.36±0.02	10.05±0.53	15.01±0.10

The proximate composition in the hydrolysates was measured as percentage composition of each constituent in the sample (Figure 27). After hydrolyzation, the hydrolysates from Batch B had an insignificant higher protein content (around 76%) compared to Batch A (around 70%). This might

be due to varying chemical constituent, as batch B samples were more refined in texture after the freeze drying. No significant difference was observed in the protein content between the different enzymes used for hydrolysis. Fish protein hydrolysate generally has a protein content in the range 60%-90% [111, 122, 123], the protein content of the mesopelagic protein hydrolysate shows a value in the middle of this range, indicating it a proteinaceous fish. As seen from the (Figure 45), fish constitute lesser fraction to average protein daily intake in g/day/person throughout the world, however its high protein and energy content. Therefore, fish, especially from the mesopelagic zone hold a high prospect in supplementing human nutrition and meeting the average daily protein intake (US RDA of 0.8g/KG body weight), due to it high protein content.

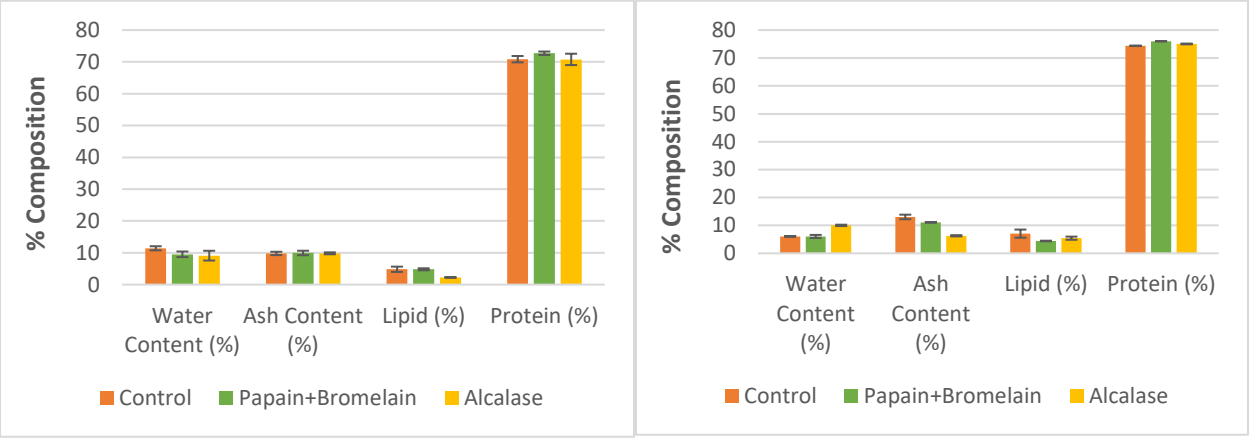

Figure 27: A: Proximate composition of protein hydrolysate from batch A (krill & pearlsides), result are presented as average (n=4) (+/- stdev). B: Proximate composition of protein hydrolysate from batch B (pearlsides), result are presented as average (n=2) (+/- stdev). Control, raw materials hydrolyzed without enzyme; papain+bromelain, raw materials hydrolyzed with these two enzymes; alcalase, raw materials hydrolyzed with enzyme alcalase.

The result from the analysis of batch A and batch B protein hydrolysate shows that lipid content varies between 2%-5%, water content between 9%-12%, and ash content between 9%-10%. The batch B hydrolysate has lower lipids and water content, and higher ash content compared to Batch A. It was expected for batch A to have the highest ash content as seen in the raw materials (Table 4) however, it can be assumed that significant portion of the minerals has been filtered out during hydrolysate preparation in batch A. Several studies have highlighted the fat content of the fish protein hydrolysate to be <5% [124], water content <10% [124], and ash content to be between 0.45% to 27% [124]. Generally, the proximate composition of mesopelagic fish protein hydrolysate recorded in the analysis falls within this expected range of proximate composition of fish protein hydrolysate.

3.2 Total amino acid composition

The total amino acid composition of batch A and batch B raw materials analyzed (Table 5). In the raw material A, the most abundant amino acids are glutamate, aspartate, lysin, leucine, and glycine/arginine (10.1, 7.5, 5.9, 5.5, and 5.2 respectively). The amino acids with the highest content in raw material B were glutamate, aspartate, lysin, leucin and glycine/arginine with the composition (7.8, 5.5, 4.6, 4.3, and 3.6 respectively). Both batches' results reiterate the high level of glutamate, aspartate, lysin, leucine, and glycine in mesopelagic fish. Furthermore, compared to batch B, these five amino acids are more abundant in batch A, except in the case of glycine/arginine, which is high in batch B.

Table 5: Total amino compostion of batch A and batch B raw materials, result are presented as average (n=3) (+/- stdev).

Total Amino Acids	Raw Materials A	Raw Materials B
	average amount mg/g sample	average amount mg/g sample
Asp	7.55±0.95	5.57±0.32
Glu	10.19±1.08	7.81±0.53
Asn	0.00±0.00	0.03±0.05
His	1.52±0.14	1.13±0.12
Ser	3.62±0.41	2.79±0.19
Gln	0.00±0.00	0.00±0.00
Gly/Arg	5.20±0.59	3.61±0.31
Thr	3.25±0.38	2.63±0.15
Ala	4.39±0.28	3.55±0.19
Tyr	2.52±0.44	1.88±0.36
Met	0.94±0.28	1.50±0.22
Val	3.26±0.22	2.70±0.19
Phe	3.14±0.33	2.44±0.18
Ile	2.95±0.30	2.28±0.19
Leu	5.54±0.58	4.39±0.27
Lys	5.95±0.36	4.60±0.28

Asp, aspartate; Glu, glutamate; Asn, asparagine; His, histidine; Ser, serine; Gln, glutamine; Gly, glycine; Arg, arginine; Thr, threonine; Ala, alanine; Tyr, tyrosine; Met, methionine; Val, valine; Phe, phenylalanine; Ile, isoleucine; Leu, leucine; Lys, lysine.

The analysis of total amino acid composition in Batch A and batch B protein hydrolysates are presented in the (Figure 28). For the batch A, the highest level of each amino acids varies between the control and alcalase hydrolysate. Highest composition of total amino acid in batch A is glutamate with concentration of 46 mg/g, followed by aspartate with composition of 33 mg/g, then lysine with the value of 32 mg/g. In the batch B, sample hydrolyzed with papain and bromelain contain highest amount of each detected amino acids. Batch B has highest level of glutamate with value of 66.02 mg/g, followed by aspartate with the value of 43 mg/g, then lysine with the value of 41 mg/g hydrolysate. Compared to batch A, batch B contain slightly higher amount of all detected amino acid measured in mg/g hydrolysate. Overall, the total amino acids content indicated that glutamate and aspartate are more abundant than other amino acids in the mesopelagic fish protein hydrolysate of batch A and B. These two closely related amino acids were known to be involved in nervous system transmission and plasticity [125].

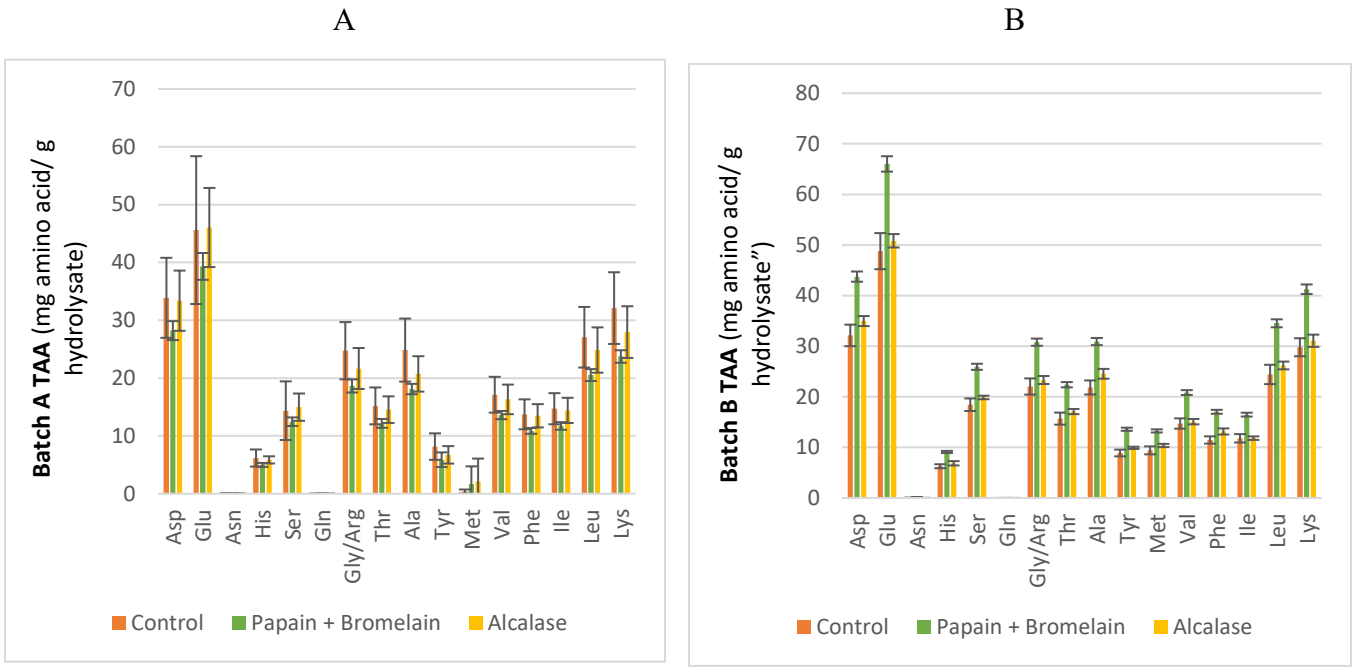

Figure 28: A: Total amino acid compostion of protein hydrolysate from batch A (krill and pearlside), result are presented as average (n=4) (+/- stdev). B: Total amino compostion of protein hydrolysate from batch B (pearlsides), result are presented as average (n=2) (+/- stdev). Control, raw materials hydrolyzed without enzyme; papain+bromelain, raw materials hydrolyzed with these two enzymes; alcalase, raw materials hydrolyzed with enzyme alcalase; TAA, total amino acid.

3.3 Free amino acid composition

The free amino acid composition of batch A and batch B of the raw materials (Table 6) was compared. In the batch A, the five most dominant free amino acid are glycine/arginine, lysine, alanine, leucine, and aspartate with a composition of 0.6, 0.4, 0.32, 0.21, and 0.14, respectively. This trend was similar for batch B, with 0.03 mg/g glycine/arginine, 0.03 mg/g leucine, and 0.02 mg/g lysine. Free amino acids are more readily absorbed upon ingestion, resulting into higher postprandial amino acid availability, and making its integration into proteins faster as compared to amino acid within a protein [126]. Therefore, conditional essential amino acids like glycine/arginine, and essential amino acid like lysine and leucine which are abundant in the free state is a promising nutritional attribute of mesopelagic fish.

Table 6: Free amino compostion of batch A and batch B raw materials, result are presented as average (n=3) (+/- stdev).

Free Amino Acids	Raw Materials A	Raw Materials B
	Average amount mg/g sample	Average amount mg/g sample
Asp	0.14±0.02	0.02±0.01
Glu	0.02±0.01	0.01±0.01
Asn	0.04±0.00	0.00±0.00
His	0.06±0.01	0.01±0.00
Ser	0.14±0.02	0.02±0.01
Gln	0.10±0.01	0.01±0.00
Gly/Arg	0.66±0.03	0.03±0.00
Thr	0.14±0.02	0.02±0.01
Ala	0.32±0.02	0.02±0.01
Tyr	0.11±0.01	0.01±0.01
Met	0.09±0.01	0.01±0.00
Val	0.14±0.01	0.01±0.00
Phe	0.13±0.02	0.02±0.00
Ile	0.10±0.01	0.01±0.00
Leu	0.21±0.03	0.03±0.00
Lys	0.43±0.02	0.02±0.01

Asp, aspartate; Glu, glutamate; Asn, asparagine; His, histidine; Ser, serine; Gln, glutamine; Gly, glycine; Arg, arginine; Thr, threonine; Ala, alanine; Tyr, tyrosine; Met, methionine; Val, valine; Phe, phenylalanine; Ile, isoleucine; Leu, leucine; Lys, lysine.

The free amino acid composition measured in mg/g sample of batch A and batch B protein hydrolysate is illustrated in the (Figure 29). In both batches, there was highest content of lysine, followed by leucine, then glycine and arginine. The lysine content was got as high as 23 mg/g hydrolysate without enzyme (Control) in Batch A. The leucine composition also had a high value of 18 mg/g hydrolysate without enzyme (Control) of Batch A. The glycine/arginine composition was high as 17 mg/g sample in the Batch A sample hydrolyzed with papain and bromelain. The presence of other free amino acid existed withing the range of 2-17mg/g, with alanine having highest value of 16.3 mg/g and 10.5 mg/g in the batches A and batch B respectively.

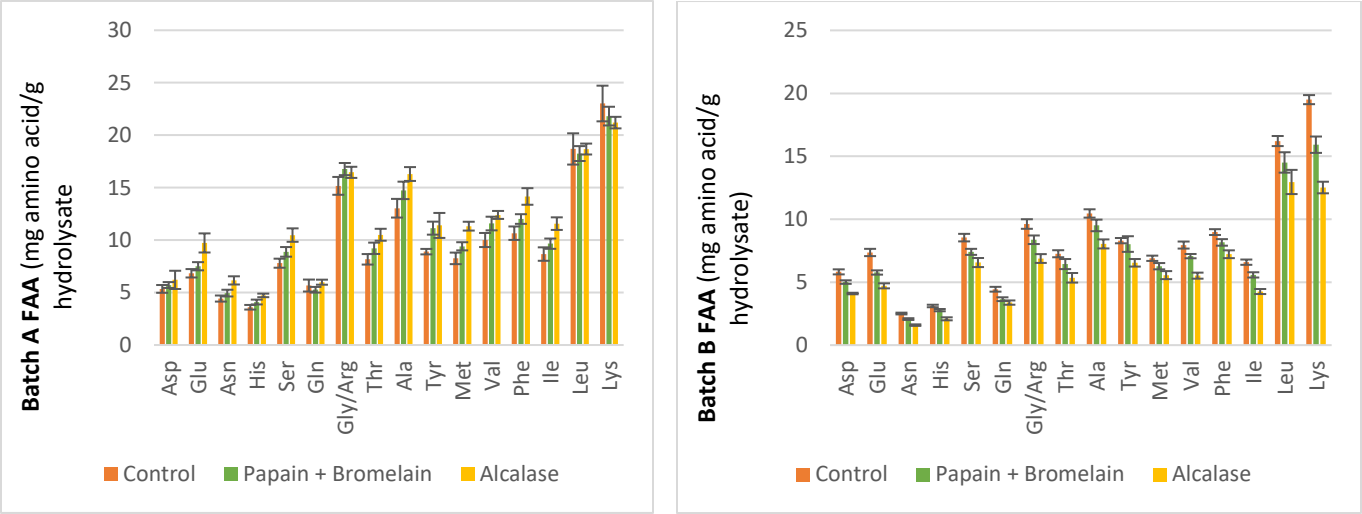

Figure 29: A: Free amino compostion of protein hydrolysate from batch A (krill and pearlside), result are presented as average (n=4) (+/- stdev). B: Free amino compostion of protein hydrolysate from batch B (pearlsides), result are presented as average (n=2) (+/- stdev). Control, raw materials hydrolyzed without enzyme; papain+bromelain, raw materials hydrolyzed with these two enzymes; alcalase, raw materials hydrolyzed with enzyme alcalase; FAA, free amino acid.

Essential amino acids are assimilated in the human body from the food source. Since they cannot be synthesized *de novo* in the human body, they are important in human nutrition, and play vital role in curbing malnutrition and stunting in children. At low serum level of these amino essential amino acids, the mTORC1 gene, which stimulate protein translation, will be repressed and the synthesis of protein and lipid will be stall. Studies have shown that the hydrolysis of marine fish yield hydrolysate with high concentration of lysine, leucine, and glutamate in their free amino state available for absorption [127]. Also, protein hydrolysate yield smaller proteins that are precursor to FAA. [128]. The average daily requirements of these essential amino acid are listed in (Appendix B), (Table 8). The high concentration of lysin, leucine, arginine, glycine, glutamate which are coherent in Batch A and B poses a possible utilization of refined protein hydrolysate from these mesopelagic fish for nutritional supplement [10].

It has been shown that hydrophobic amino acids and amino acids with sulphur and nitrogen in the side chain have tendency of quickly undergo oxidation. This is because of the present of lone pair of electrons available to be attacked by antioxidant [129]. These amino acids inundated the free and the total amino acids detected, and therefore it is safe to assume they confer antioxidant capabilities of the protein hydrolysate.

3.4 Bioactivities

In addition to free amino acid content, bioactivities of protein hydrolysate have been linked to size of the peptides. Most antioxidative peptide have a molecular weight less than 1kDa, and usually contain higher content of hydrophobic amino acids [130]. In addition, Ala-Tyr dipeptides have been shown to possess novel antioxidative abilities [131]. ACE inhibitory peptides have been found to have a molecular weight in the range of 0.1kDa – 2kDa, with prevalent peptide sequence of Val-Ser-Gln-Leu-Thr-Arg, Met-Glu-Val-Phe-Val-Pro, Val-Ser-Gln-Leu-Thr-Arg [38]. Example of relevant antioxidant and anti-hypertensive are displayed in the (Table 2)

3.4.1 The FRAP assay

The FRAP assay quantitatively measured the ability of the protein hydrolysate to reduce Fe^{3+} to Fe^{2+}, and the values is expressed as Trolox equivalence (TE) and represent the antioxidant activities. The result from the FRAP assay indicated the protein hydrolysate of batch A (pearlside and krill) has higher antioxidant power as compared to batch B (pearlside) protein hydrolysate (Figure 30)

The antioxidant ability of batch A was approximately double that of batch B for all the samples analysed. The sample hydrolysed with papain + bromelain showed highest activities in both batches with batch A having value of 1389 µmol TE/g, while batch B had a value of 543 µmol TE/g. The bioactivities of the raw materials was the lowest followed by the control expereiment. Hydrolysate of alcalase and papain + bromelain show equall antioxidative properties (Figure 30).

Overall, the FRAP result of the batch A protein hydrolysate were higher than most marine fish protein hydrolysate reported in the literature. Protein hydrolysate from head and backbone of saithe (*Pollachius virens*) have been documented to have a FRAP value of 5.16 µmol/g and 12.57 µmol/g respectively [132]. On the other hands, the FRAP value of batch B samples were slightly higher than that reported for Tilapia viscera protein hydrolysate during the vitro gastrointestinal digesting, whose values varied from 75 µmol/g to 300 µmol/g. The increasingly large value of the batch A FRAP value might be due the mixture of Krill and Pearlside in it's sample which confer higher bioactivities as compared to batch B containing only pearlsides (Figure 30).

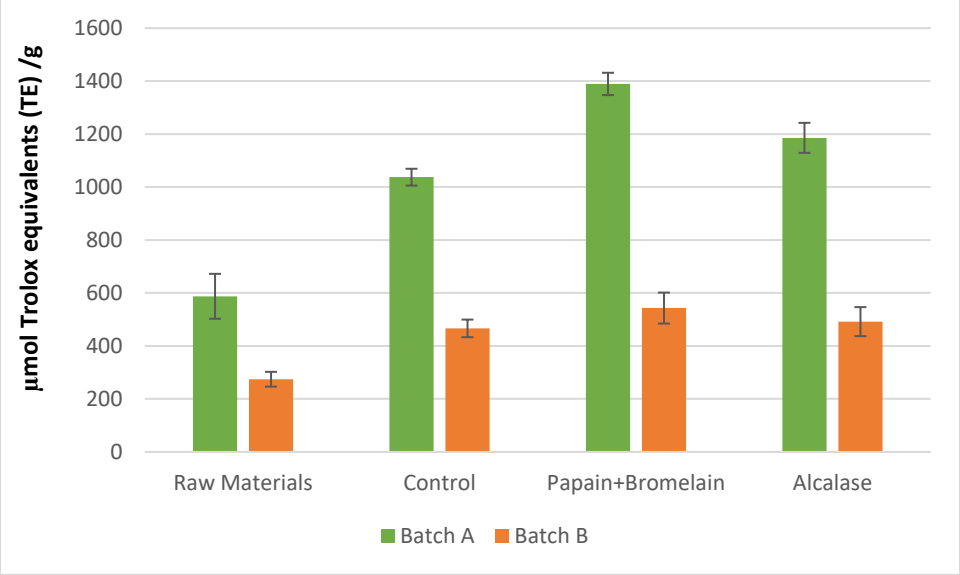

Figure 30: Ferric Reducing Antioxidant Power (FRAP) of raw samples or hydrolysates of two different batches of mesopelagic fish, batch A and batch B. Result are presented as average (n=2) (+/- stdev) of the raw materials and (n=4) (+/- stdev) of the hydrolyzed sample Control, raw materials hydrolyzed without enzyme; papain+bromelain, raw materials hydrolyzed with these two enzymes; alcalase, raw materials hydrolyzed with enzyme alcalase; Batch A, krill and pearlside; Batch B, pearlside.

3.4.2 The ABTS assay

The ABTS bioassay works by generating $ABTS^{+}$ which is a stable radical cation suitable for measuring the total antioxidant capacity of natural product by mapping how effectively the radical cation is being reduced, and then compared with propyl gallate as standard [133]. Batch B exhibited significantly higher ABTS activity compared to that of batch A (2200 PG and 1100 PG, respectively for the raw material), with sample of papain + bromelain hydrolysis insigficantly higher. There is insignificant difference in the ABTS activity of protein hydrolysate in batch A and batch B. However, papain + bromelain hydrolysate in was slightly higher both baches, indicating the enzyme was effective in the cleavage of the peptide bond into smaller peptides which thus confer slightly higher antioxidant acitivities (Figure 31).

The propyl gallate equivalence of the batch B protein hydrolysates existed around 6000 µmol/g. The batch B raw materials however have an antioxidant value around 2000 µmol PG/g. It was expected that the antioxidant activities of the mix mesopelagic sample of batch A to be higher than that of one fish sample of batch B. As opposed to the result from the FRAP assay, the ABTS result of the batch A was generally lower than that of batch B sample, and existed within the range of 4000 PG/g for the protein hydrolysate, and 1000 PG/g for the raw materials. Generally, batch A ABTS result is half of what is obtained in the batch B analysis (Figure 31).

The reported ABTS antioxidant value for some marine fishes are generally lower than than experimental result of the batch A and B. The protein hydrolysate from head and backbone of saithe (*Pollachius virens*) has a ABTS antioxidant value of 58.33 ± 0.64 µmol/g and 63.57 ± 1.20 µmol/g respectively [132], the ABTS result of permeate-based protein hydrolysate from atlantic salmon shows a value of about 100 µmol/g [134]. The ABTS result of the mesopelgic fish protein hydrolysate is generally high, expecially in batch B, and might have potential to quench oxidation cascade in living cells (Figure 31).

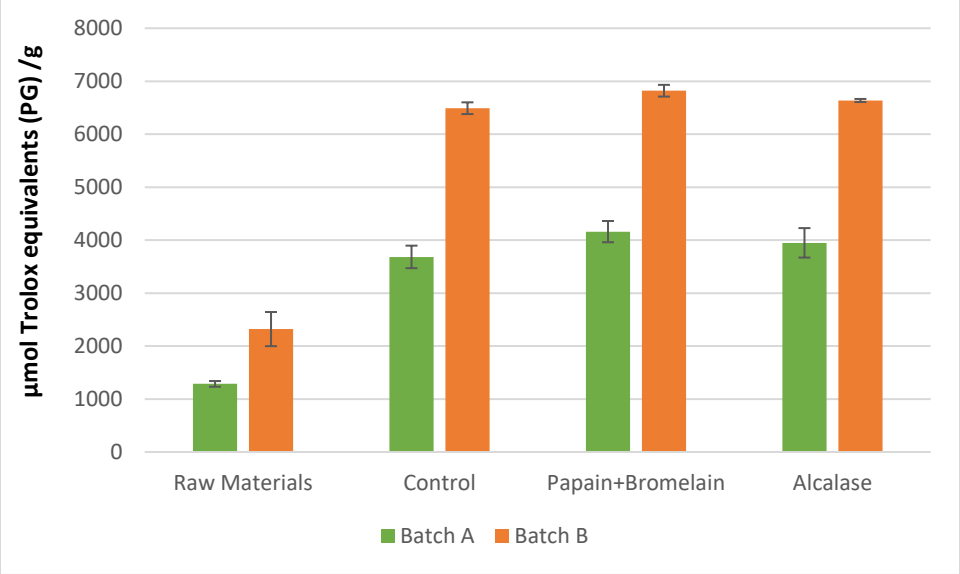

Figure 31: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) activity of sample or hydrolysates of two different batches of mesopelagic fish, batch A and batch B. Result are presented as average (n=2) (+/- stdev) of the raw materials and (n=4) (+/- stdev) of the hydrolyzed sample. Control, raw materials hydrolyzed without enzyme; papain+bromelain, raw materials hydrolyzed with these two enzymes; alcalase, raw materials hydrolyzed with enzyme alcalase; Batch A, krill and pearlside; Batch B, pearlside

3.4.3 The DPPH assay

The DPPH assay measure the ability of the food sample to exhibit antioxidant effect in a concentration dependent manner, in which the DPPH is reduced to diphenyl picryl hydrazine. The DPPH activities of batch A sample is higher than that of the batch B for raw material and protein hydrolysates. The value of batch A DPPH activity measured was signicantly higher than that of batch B and fall within the range of 1500 µmol PG/g to 2000 µmol PG/g with sample from papain + bromelain hydrolysis being the highest in batch A and alcalase based hydrolysate in the batch B. The batch B DPPH activities on the other hands exhibited 400 µmol PG/g to 750 µmol PG/g

for the freezdried protein hydrolysates. The DPPH activities of both batches raw materials is significantly lower compared to FRAP and ABTS result. Unlike the FRAP and ABTS bioactivities, the control protein hydrolysate displayed higher bioactivities than the sample from alcalase in the batch A. The higher DPPH scanvenging activities reported for both batches of mesopelagic fish is higher than what was reported in literature for marine fish like Salmon [135]. Like other bioassay result, higher DPPH is also an indication of the ability of the marine mesopelagic fish protein hydrolysate to inhibit free radical reaction (Figure 32).

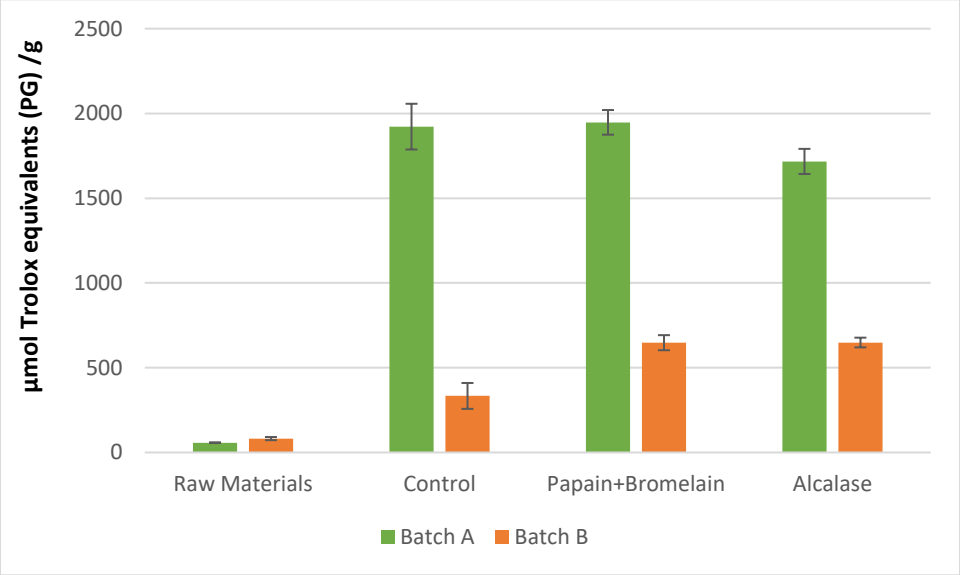

Figure 32: 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity of samples or hydrolysates of two different batches of mesopelagic fish, batch A and batch B. Result are presented as average (n=2) (+/- stdev) of the raw materials and (n=4) (+/- stdev) of the hydrolyzed sample Control, raw materials hydrolyzed without enzyme; papain+bromelain, raw materials hydrolyzed with these two enzymes; alcalase, raw materials hydrolyzed with enzyme alcalase; Batch A, krill and pearlside; Batch B, pearlside

3.4.4 The ORAC assay

The ORAC assay employed the kinetics means to determine the antioxidant capacity of the natural product. ORAC is measured as trolox equivalence in µmol TE/g. Raw material and hydrolysates of batch A exhibited significantly higher ORAC compared to those from batch B. The value of the batch A protein hydrolysates exhibited ORAC value within the range of 400 µmol TE/g to 600 µmol TE/g, and the sample hydrolysed with papain + bromelain showed the highest activities which is about 600 µmol TE/g. The ORAC assay result of the control sample in the batch A is higher than the alcalse hydrolysed protein hydrolysate, however, the standard deviation of control sample of the batch A is very high. The batch B protein hydrolysates exhibited 20 µmol TE/g to

50 µmol TE/g ORAC, which is very small when compared to the batch A result. The sample were ran at different time and day and might have acounted for such big difference. As expected, the ORAC result on the raw materials of both batches is low compared to the hydrolysates. Interesting comparism from the literature is the protein hydrolysate from head and backbone of saithe (*Pollachius virens*) which has an ORAC antioxidant value of 380.50 ± 36.12 µmol TE/g and 557.22 ± 72.47 µmol TE/g respectively, close to the results obtained for the batch A protein hydrolysates (Figure 33).

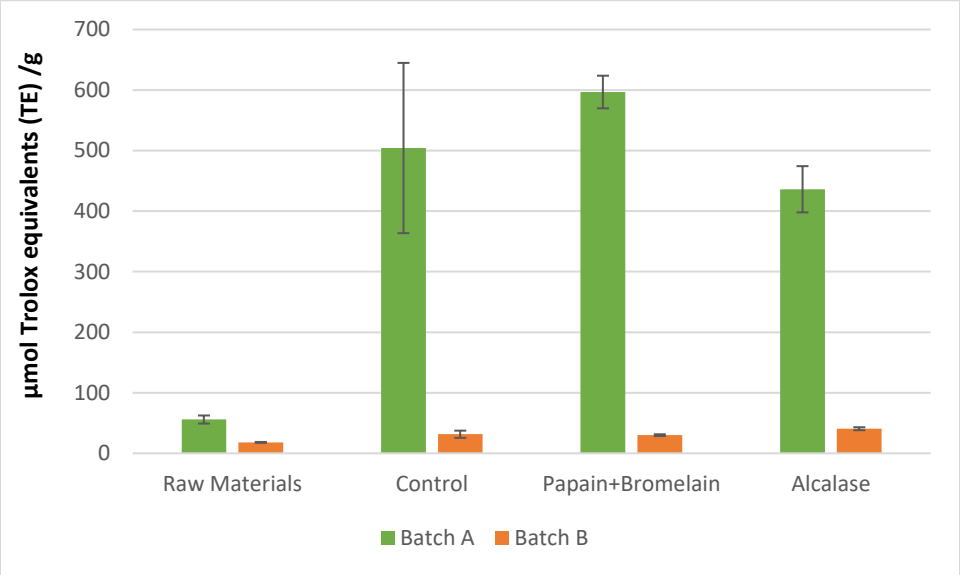

Figure 33: Oxygen Radical Absorbance Capacity (ORAC) of samples or hydrolysates of two different batches of mesopelagic fish, batch A and batch B. Result are presented as average (n=2) (+/- stdev) of the raw materials and (n=4) (+/- stdev) of the hydrolyzed sample Control, raw materials hydrolyzed without enzyme; papain+bromelain, raw materials hydrolyzed with these two enzymes; alcalase, raw materials hydrolyzed with enzyme alcalase; Batch A, krill and pearlside; Batch B, pearlside.

The ORAC score is used to evaluate the antioxidant activities in food. About 25% of energy generted in the mitochondrial is sub-optimally utilized [136], and this energy is believed to drive oxidative stress and free radical generation. Therefore, ORAC value of about 3200 µmol TE would optimally salvage postprandial oxidative stress [137]. Furthermore, assuming there exist equivalence connection between energy intake and antioxidant requirement, then we can calculate dietary antioxidant per energy consumed. Antioxidant capacity requirements for a person consuming 2500 kcal per day (optimum energy requirement) are estimated to be 11,500 µmol TE. Antioxidant capacity as ORAC intakes of 5–18 µmol TE per day are likely achievable with the right combination of strong antioxidant meals [137]. In addition, an epidemiological study back up the idea that dietary ORAC intakes exceeding 10,000 µmol TE per day (as measured by the peroxyl radical in the ORAC) are linked to a lower risk of hypertension, all-cause mortality, cerebral infarction, endometrial cancer, and stroke [137].

3.5 ACE inhibotory activity

The result of ACE assay shows that alcalase based protein hydrolysate in batch A and batch B have the highest inhibitory effect, with the IC_{50} value of 10.05 and 7.94 respectively (Table 7). This is because, compared to other protein hydrolysate, alcalase hydrolysate required minimum amount of sample to halve the ACE enzyme. The result is in a way close the antioxidant assay result (Figure 30-33), where alcalase protein hydrolysates have shown closely higher activity after the papain+bromelain hydrolysate. Since smaller peptide has been attributed to have high ACE inhibitory effect (Table 2), this result is as expected for the enzyme-based hydrolysate, which possess highest quantity of smaller peptide. However, this result is not reliable, because of large standard deviation as seen in the IC_{50} value of control A and papain+bromelain A protein hydrolysates. Also, second parallel of alcalase A showed large value out of the range and was later discarded. Furthermore, the ACE method is still developing, and further refinement is needed to ensure the viability of the result. Generally, the IC_{50} value for the protein hydrolysate is higher compared to the IC_{50} of < 0.2mg/ml obtained in ACE study of Northern krill and pearlside hydrolysate [26].

Table 7: Summary of ACE result for the two batches of protein hydrolysate, showing amount in mg of protein hydrolysate required to halve 1mU ACE enzyme. The result presented as an average ± standard deviation for control A and papai+bromelain A. Control, raw materials hydrolyzed without enzyme; papain+bromelain, raw materials hydrolyzed with these two enzymes; alcalase, raw materials hydrolyzed with enzyme alcalase; Batch A, krill and pearlside; Batch B, pearlside.

Batch A		
Protein hydrolysate A		IC_{50}
Control A	n=2	13.86±1.59
Papain+bromelain A	n=2	15.61±6.24
Alcalase A	n=1	10.05

Batch B		
Protein hydrolysate B		IC_{50}
Control B	n=1	13.17
Papain+bromelain B	n=1	10.49
Alcalase B	n=1	7.94

3.5 Degree of hydrolyzation

The degree of hydrolyzation measured by formyl titration is a valuable method to measure the amount of peptide bonds cleaved in the protein hydrolysate. The higher the amount of protein cleaved, the more readily available fragment of lower molecular weight peptide, which has been reported to contribute immesely to bioactivities of the protein hydrolysate [138]. From the result, in both batches of protein hydrolysate, alcalase resulted in highest degree of hydrolysation, followed by papain + bromelain. In general sense, the degree of hydrolysation result is more resonable when observing the antioxidant capacities from FRAP, ABTS, DPPH and ORAC. Sample hydrolysed with enzymes has repeatedly show higher antioxidant value in all these four assays. This means that these sample have been broken down to simple peptides which is

responsible for higher degree of hydrolyzation and hence higher bioactivities. It is interesting to see the control sample hydrolyzed only with endogenous enzyme having closely degree of hydrolyzation to both enzymatic hydrolysis. (Figure 34). On average, the overall degree of hydrolyzation for all the hydrolyzate is 30%, and this is closely related to the degree of hydrolyzation of alcalase based hydrolyzate from Tuna (*Katsuwonus pelamis*) blood, which was also 30% DH over different treatment [139]. Furthermore, the 30% average obtained for this result is slightly higher than 10% – 20% recorded for alcalase based hydrolysate obtained frame meat of striped catfish [140]. Degree of hydrolysis has been shown to increase with the pH and hours of hydrolysis as seen in hydrolysate from parrotfish which has 30% DH at pH 9 for 24 hours hydrolysis [141]. This value is averagely the same with the degree of hydrolysis of the mesopelagic fish hydrolyzed for just 1 hour at neutral pH (6.5-7.5).

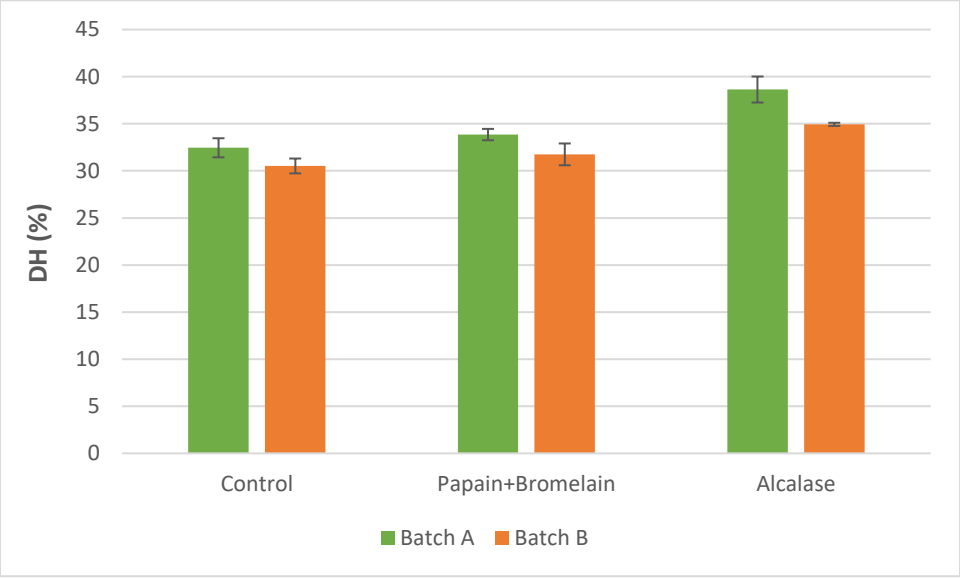

Figure 34: Degree of hydrolysation result are presented as average (n=2) (+/- stdev). Control, raw material hydrolyzed without enzyme; papain+bromelain, raw material hydrolyzed with these two enzymes; alcalase, raw material hydrolyzed with enzyme alcalase.

3.6 Protein yield

The protein yield of the hydrolysate powder was calculated based on the weight and protein content of hydrolysate powder compared to the weight and protein content of raw material. The yield expressed as protein in hydrolysate as percentage of protein in the raw materials. The result from the yield calculations showed that the alcalase hydrolysed raw materials in both batches of experimental sample have the highest yeild, indicating the efectiveness of alcalase in cleaving

peptides bonds in the raw materials. The value for alcalase based hydrolysis for batch A and B are 29% raw materials and 26% raw materials (Figure 35).

The papain + bromelain based hydrolysate also resulted in a considerable high yeild next to the alcalase, and the value for the batch A and B 26% and 24% respectively. The control sample which is hydrolysed without enzyme show less yeild result of 23% and 11% for batch A and B experimental sample respectively. The yeild of the control sample is lower in both batches due to the sub-optimal cleavage of the peptide bond in the raw maeterial, which made it difficult to recovered most of the protein out in the hydrolysate. For batch A there is a slight increased protein yield with enzymes (going from control to alcalase), but for batch A the difference is large. The sample of the batch B was not analysed in parallel/bioreactors due to limited available sample, but the procedure was strictly followed like in batch A analysis which made the result reliable enough as a rough estimate (Figure 35). On average, compared to the 3% average yeild reported for the alcalase based hydrolysate from tuna [139], approximately 30% yeild of the protein hydrolysate is high. Futhermore, higher yeild was observed when compared to approximately 10% average otained from alcalase based hydrolysate obtained frame meat of striped catfish [140]. Protein hydrolysate from parrotfish (Chlorurus sordidus) head yeilded 49% after hydrolysis at pH 9 for 24 hours [141], comapared to the 30% average yield of the protein hydrolysate in this result. The raw materials of mesopelagic fish was hydrolyzed under neutral pH range (6.5 – 7.5). This give a propable insight on the yeild relationship with pH. However, the average yeild in this result is still quite optimum considering that it was only hydrolyzed for 1 hour.

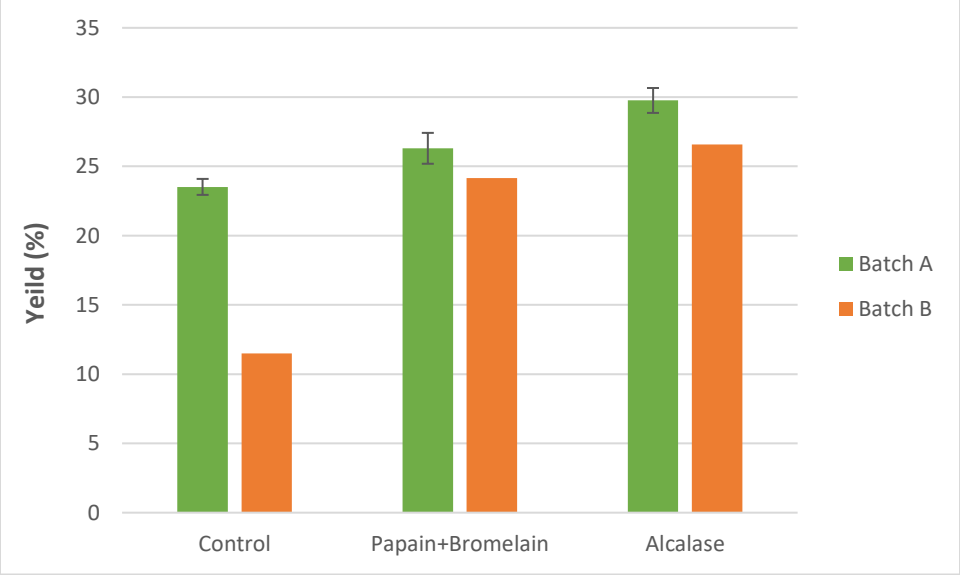

Figure 35: Yield of the protein hydrolysate expressed in percentage recovered protein per gram of the raw materials. Result are presented as average (n=2) (+/- stdev) for the Batch A, and (n=1) for the Batch B. Control, raw material hydrolyzed without enzyme; papain+bromelain, raw material hydrolyzed with these two enzymes; alcalase, raw material hydrolyzed with enzyme alcalase.

3.7 Molecular weight distribution

The molecular weight distribution from the (Figure 36) indicated the prominence of peptide fraction < 0.2kDa. Peptide fraction from the alcalase based protein hydrolysate of the batch A sample contain highest amount of < 0.2kDa peptides, with the value of about 350 mg/g protein hydrolysate. Papain + bromelain-based enzyme hydrolysate from the batch A had 315 mg/g protein hydrolysate of peptide fraction < 0.2kDa. The non-enzymatic hydrolysate of the batch B (Control B) has in its peptide sequence 306 mg/g protein hydrolysate of < 0.2kDa. The control of batch A (Control A) has a 302 mg/g protein hydrolysate of < 0.2kDa peptide segment, and this was followed by papain + bromelain (papain+bromelain B) and alcalase (Alcalase B) based hydrolysate from batch B which has 297 mg/g and 268 mg/g protein hydrolysate of < 0.2kDa peptide segment. Other peptide fragment of molecular weight > 0.2 kDa are significantly lower in comparison with the amount < 0.2 kDa in all the samples.

In general, peptides < 0.2kDa is the most abundant in all the hydrolysis, this is followed by peptide fraction of 1.0 – 0.1 kDa molecular weight. Peptide fraction of 2 – 1 kDa is the third most dominating peptide fraction of batch B, which is also partly true for the batch A, but with the exception in the alcalase based hydrolysis where 0.5 – 0.2 kDa peptides fraction took the third position. The rest of the peptides fraction, which is from 5 – 2 kDa to > 20 kDa appear in smaller quantities. The major interest has been on peptide fraction of lower molecular weight due to its attribution to bioactivities. Peptide with 0 – 3 kDa in molecular weight has been showed to have highest DPPH activities in scavenging hydroxyl (OH) free radicals [142], and the molecular weight fraction of peptides < 1kDa has generally been shown to have high antioxidant and ACE inhibitory activities [139].

The higher antioxidant capacities recorded in all the assays, particularly the enzyme-based hydrolysate can be link to the presence of an abundant low molecular weight peptide because of enzyme hydrolysis, while the raw materials and non-enzymatic hydrolysis show lower antioxidant assay result, due to less fraction of low molecular weight peptide in the sequence.

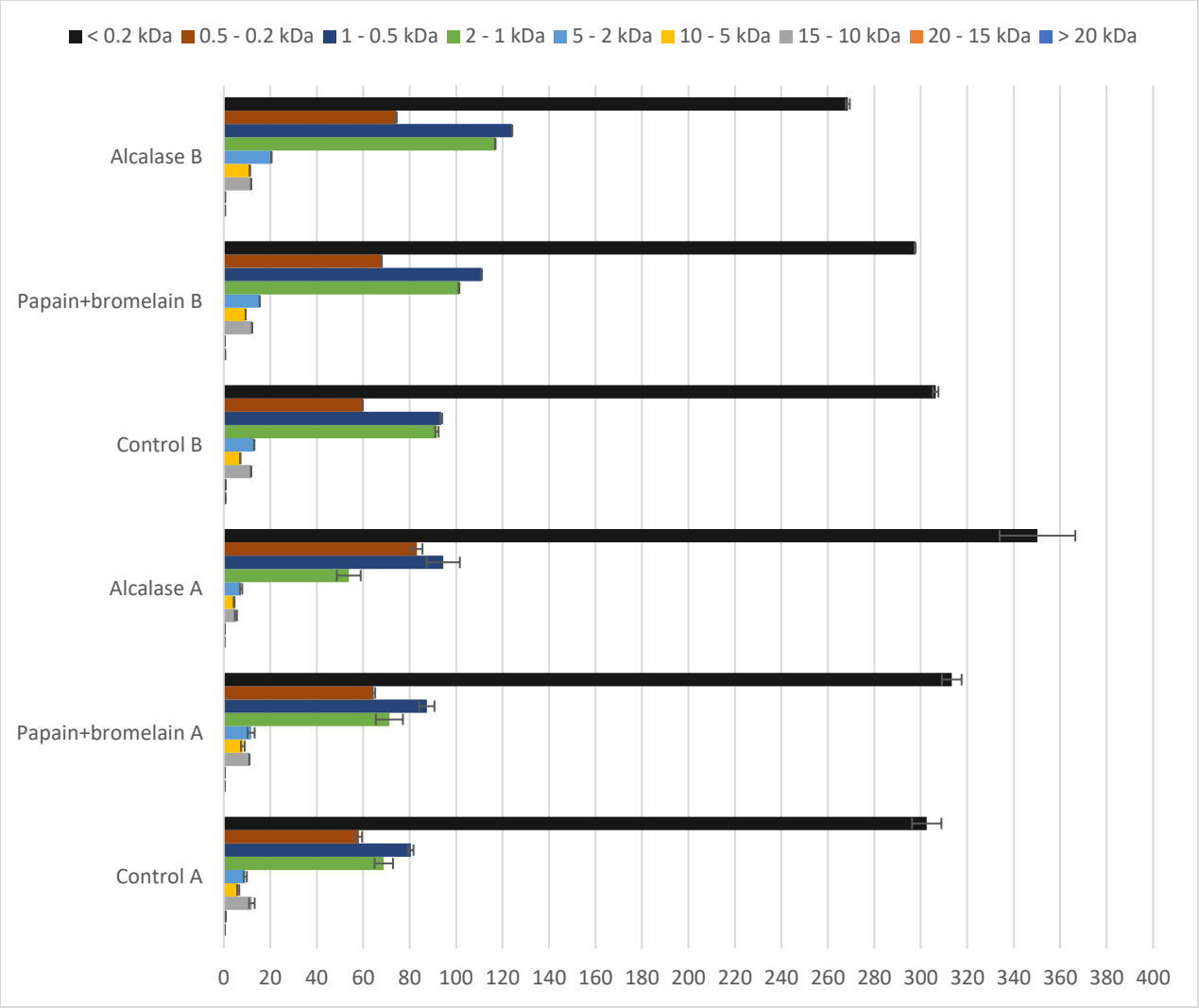

Figure 36: Molecular weight distribution of batch A and batch B protein hydrolysate powder measured as kDa (kilodalton) in mg/g protein hydrolysate powder, ($\bar{x}\pm$ SEM, n = 4) for batch A, and ($\bar{x}\pm$ SEM, n = 2) for the batch B. A, batch A; B, batch B. Control, raw materials hydrolyzed without enzyme; papain+bromelain, raw materials hydrolyzed with these two enzymes; alcalase, raw materials hydrolyzed with enzyme alcalase; Batch A, krill and pearlside; Batch B, pearlside.

4 Conclusion

The mesopelagic fish protein hydrolysate from Atlantic krill and pearlside sample mixture of batch A has an average protein content of 72%, water content of about 10%, ash content of about 10%, and lipid content of 4%. The protein hydrolysate from sample containing only pearlside (batch B) has a protein content of about 75% on average, 8% water content, 10% ash content, and 6% lipid content. The proximate composition conforms to the well-established proximate composition of the marine fish in literature [124], and high amount of protein content was an indication to nutritional benefit.

Present in the two batches of mesopelagic protein hydrolysate were the abundance of lysine, leucine, glycine and arginine, and alanine in the free amino state. These amino acids are consistent, with lysine being the most abundant. Furthermore, the total amino acids content also indicated the presence and abundance of glutamate, aspartate, and lysine in the protein hydrolysate of the mesopelagic fish. Based on the abundance of each amino acids, molecular weight and compared to the peptides sequence that have been shown to possessed bioactivity in some marine fish (Table 2) Leu-Asp-Lys tripeptide as seen in *Sphyrna lewini* muscle is suspected to be responsible of the antioxidant activity. To further established this, the molecular weight of Leu-Asp-Lys tripeptide is 374.43g/mole (0.3kDa) which fall size range of antioxidant peptides. The ACE inhibitory peptide suspected are Leu-Gly-Leu-Ser (Mw= 2.9 kDa), Phe-Leu (Mw= 0.3 kDa), present in squid gelatin and salmon muscle, respectively.

The enzyme-based hydrolysate shows highest bioactivities. Also, the control sample from both batches has shown optimum bioactivities which values are not far off from that of enzymatic hydrolysis. This is further confirmed by the yield and degree of hydrolyzation result of the control which are also close to the enzyme-based hydrolysate. Papain+bromelain-based hydrolysate has repeatedly proved to have highest antioxidant capacities measured in µmol/g sample in the FRAP, ORAC, DPPH, and ABTS assays. Although with numerous uncertainties in the result, the alcalase based hydrolysate has showed highest ACE inhibitory value for both batches of protein hydrolysate

The yield of alcalase based hydrolysate was the highest, this was followed by papain + bromelain-based hydrolysate. There is an insignificant different in yield and degree of hydrolyzation when compared all the enzyme-based hydrolysates. However, it was intriguing to see the yield value of the control sample remarkably close to the treatment sample, meaning the endogenous enzyme in the mesopelagic fish tissue were also effective in cleaving the peptide bonds, and this might be exploited in an industrial setting with little to no exogenous enzyme to maximally increase the protein production yield.

The higher antioxidant capacities of the mesopelagic fish protein hydrolysate of batch A and B as compared to their control and raw materials sample can be trace to the abundant of low molecular weight peptides (< 0.2 kDa) upon hydrolysis. Other fraction that also contributed to the antioxidant abilities are the peptides in the range of 0.5 – 2 kDa. Higher molecular weight peptides were present in minimal quantities and in all the sample of the two batches.

5 Future outlook

There is deterrence in the consumption of krill due to its salty nature, toxic fluoride, and heavy metal content. This was seen in vacuoles irregularities of salmon fed with krill as compared to the conventional fishmeal [31]. In addition to bony structure and small size, mesopelagic fish protein hydrolysate tendency to autolyze rapidly and its bitterness has render it unsuitable for human consumption. As of now, mesopelagic fish protein hydrolysate can be processed into nutraceutical ingredient that can serve as nutrient fortification in human, and to formulate animal feed, which can support the aquaculture growth.

Mesopelagic fish protein hydrolysates have shown a promising nutritional and therapeutic qualities both in terms of proximate composition and bioactivity measurement. However, there is a need for human trial to establish the safe dosage. Cutting-edge technology, and high-throughput sequencing techniques that can refine peptide sequence and monitor the mechanism by which they act in the oxidative and hypertensive pathway will help to identify a unique peptide sequence for drug designing. Availability of several debittering molecule will widen the scope of nutritional application of marine mesopelagic fish protein hydrolysate. Moreso, to fully maximized the application of marine mesopelagic fish in the aquaculture sector, there is a need to decontaminate the toxic fluorine and heavy metal content for the proper health of the farmed fish.

Recent development to incorporate microwave irradiation while performing the enzyme assisted method known as microwave-assisted enzymatic extraction (MAEE) is expected to address the challenges of low yield output, functional properties, and improved bioactivities of the product associated with enzymatic based approach [38].

Due to its current high instability, the ACE procedure need to be developed. This will open a possibility to accurate know the protein hydrolysate with highest ACE inhibitory effect and can thus further be compared to the peptide sequencing result, which makes it easy to draw the conclusion on small bioactive peptides.

6 Reference

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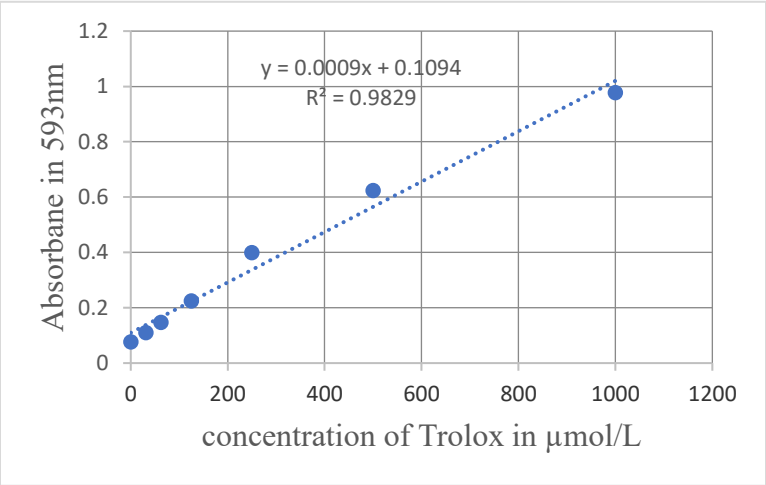
7 Appendix

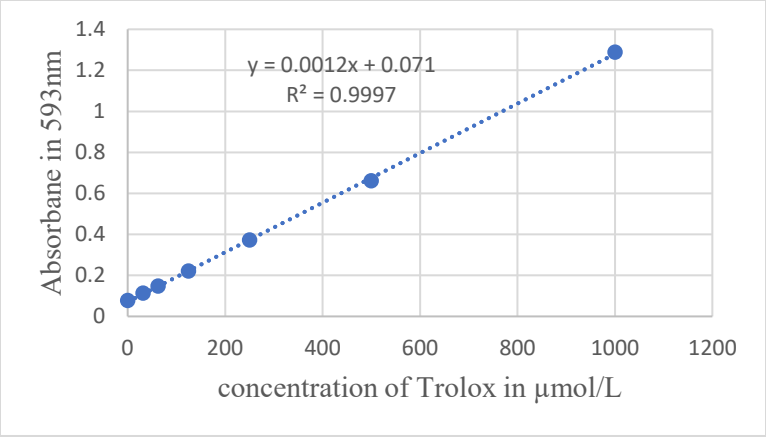
A Standard curve for antioxidant measurement

The standard curve used to measure the antioxidant status of the protein hydrolysate and the raw materials in sample A and B is presented from figure 37 – 44.



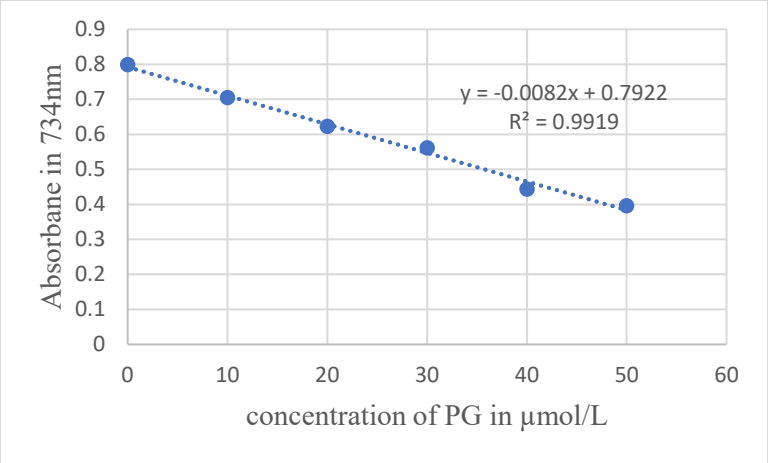
The concentration of FRAP in 1:1000 diliution µmol/L (X) was calculated by using the equation: y = 0.0009x + 0.1094, where y is the absorbance of the sample, -0.0009 is the slope of the standard curve, and 0.1094 is the intercept on the y-axis. The obtained value of X was used to calculate Concentration of samples in µmol/g

Figure 37: Batch A (Krilll+Pearlside) FRAP standard curve



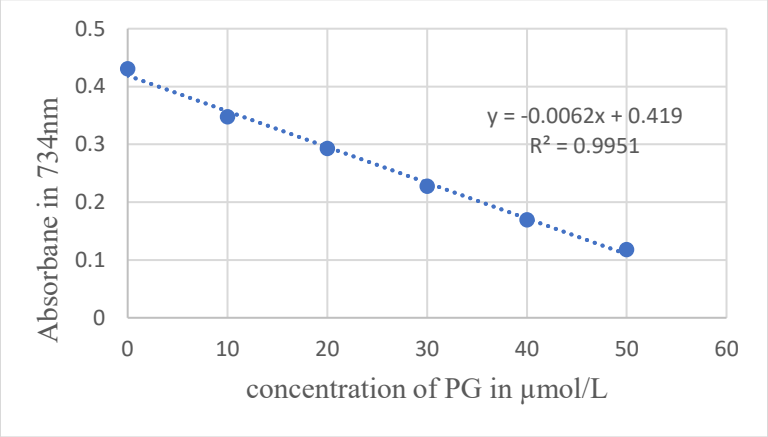
The concentration of FRAP in 1:1000 diliution µmol/L (X) was calculated by using the equation: y = 0.0012x + 0.071, where y is the absorbance of the sample, 0.0012 is the slope of the standard curve, and 0.071 is the intercept on the y-axis. The obtained value of X was used to calculate Concentration of samples in µmol/g

Figure 38: Batch B (Pearlside) FRAP standard curve



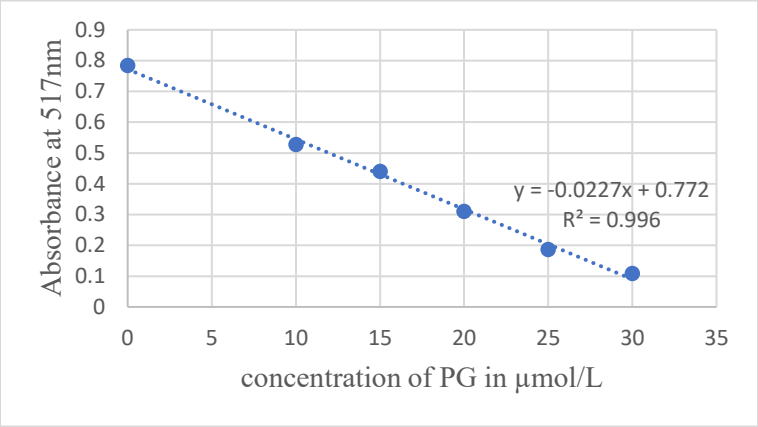
The concentration of ABTS in 1:1000 diliution µmol/L (X) was calculated by using the equation: y = -0.0082x + 0.7922, where y is the absorbance of the sample, -0.0082 is the slope of the standard curve, and 0.7922 is the intercept on the y-axis. The obtained value of X was used to calculate Concentration of samples in µmol/g

Figure 39: Pearlside + Krill (Batch A) ABTS standard curve



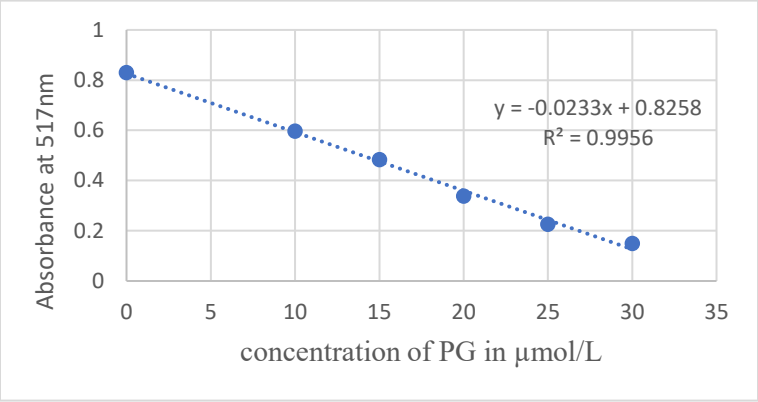
The concentration of ABTS in 1:1000 diliution µmol/L (X) was calculated by using the equation: y = -0.0062x + 0.419, where y is the absorbance of the sample, -0.0062 is the slope of the standard curve, and 0.419 is the intercept on the y-axis. The obtained value of X was used to calculate Concentration of samples in µmol/g

Figure 40: Pearlside + Krill (Batch A) ABTS standard curve



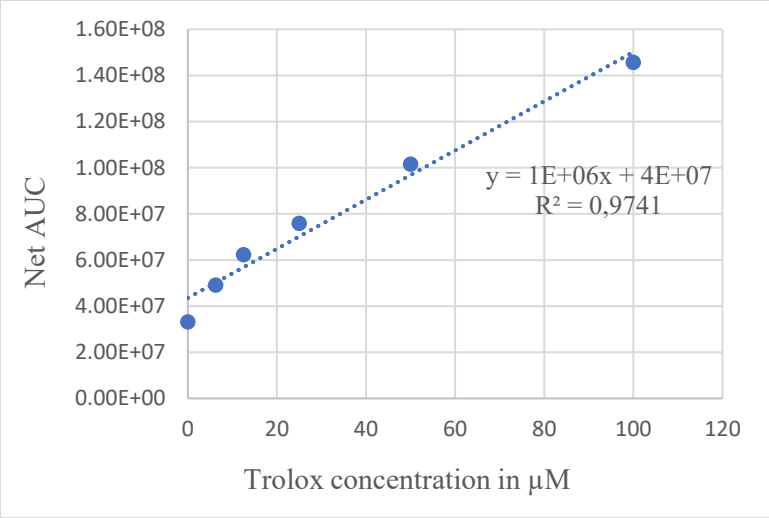
The concentration of DPPH in 1:1000 diliution µmol/L (X) was calculated by using the equation: y = -0.0227x + 0.772, where y is the absorbance of the sample, -0.0227 is the slope of the standard curve, and 0.772 is the intercept on the y-axis. The obtained value of X was used to calculate Concentration of samples in µmol/g

Figure 41: Pearlside + Krill (Batch A) DPPH standard curve



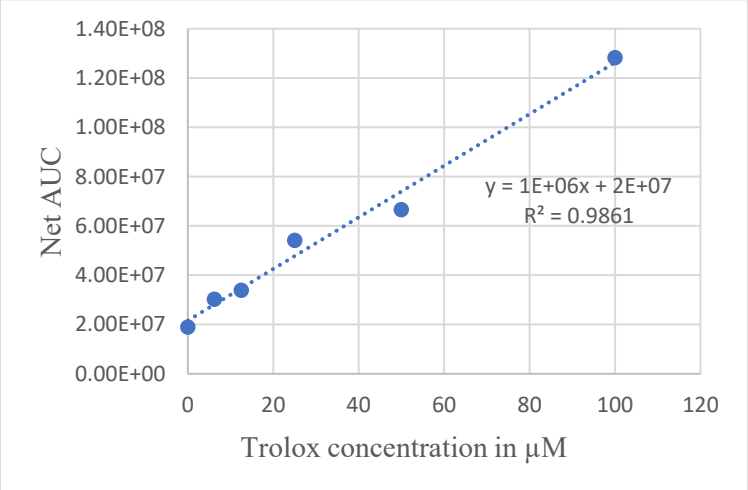
The concentration of DPPH in 1:1000 diliution µmol/L (X) was calculated by using the equation: y = -0.0233x + 0.8258, where y is the absorbance of the sample, 0.8258 is the slope of the standard curve, and 0.0233 is the intercept on the y-axis. The obtained value of X was used to calculate Concentration of samples in µmol/g

Figure 42: Pearlside (Batch B DPPH) standard curve



The concentration of ORAC in 1:10,000 diliution µM (X) was calculated by using the equation: $y = 10^{-6}x + 4*10^{-7}$, where y is the absorbance of the sample, 10^{-6} is the slope of the standard curve, and $4*10^{-7}$ is the intercept on the y-axis. The obtained value of X was used to calculate Concentration of samples in µmol/g

Figure 43: Batch A (Krill + Pearlside) ORAC Standard curve.



The concentration of ORAC in 1:10,000 diliution µM (X) was calculated by using the equation: $y = 10^{-6}x+2*10^{-7}$, where y is the absorbance of the sample, 10^{-6} is the slope of the standard curve, and $2*10^{-7}$ is the intercept on the y-axis. The obtained value of X was used to calculate Concentration of samples in µmol/g

Figure 44: Batch B (Pearlside) ORAC standard curve.

B Protein and essential amino acid

Figure 45. displayed the protein intake over the time by country status, this was used a background to emphasize that, however marine fish possess great nutritional quality, food from ocean is consumed less throughout the world. Table 7. showed the essential amino acids requirement per day by age group and was compare mesopelagic fish protein hydrolysate to know what quantity can can meet this requirement.

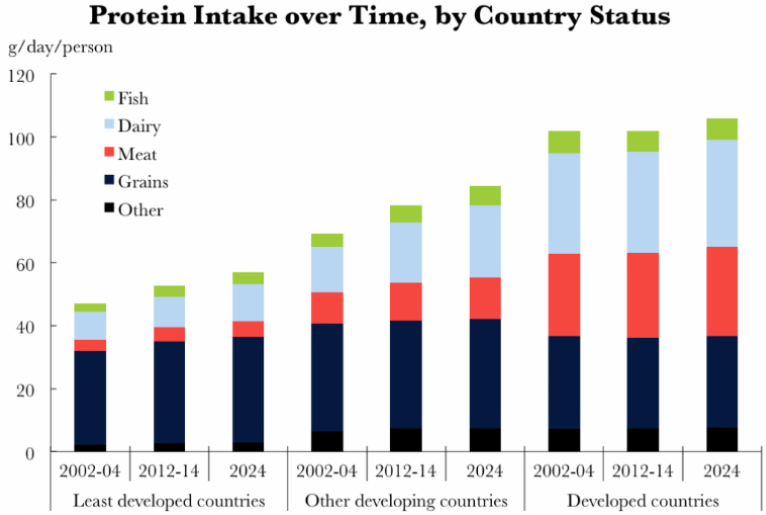


Figure 45: Food source of protein intake g/day/person over the time and by country statues [143]

Table 8: Essential amino acids requirement, mg/kg per day, by age group [144]

	Requirements, mg/kg per day, by age group			
Amino Acid	**Infants, Age 3–4 mo[b]**	**Children, Age ~2 yr[c]**	**Children, Age 10–12 yr[d]**	**Adults[e]**
Histidine	28	?	?	8–12
Isoleucine	70	31	28	10
Leucine	161	73	42	14
Lysine	103	64	44	12
Methionine plus cystine	58	27	22	13
Phenylalanine plus tyrosine	125	69	22	14
Threonine	87	37	28	7
Tryptophan	17	12.5	3.3	3.5
Valine	93	38	25	10
Total without histidine	714	352	214	84

C Molecular weight distribution

The figure shows the molecular weight distribution chromatogram of batch A and B sample, each color is used to depict bioreactor parallel of hydrolysis or parallel. Figure show overlapping chromatogram of the batch a and batch B protein hydrolysate

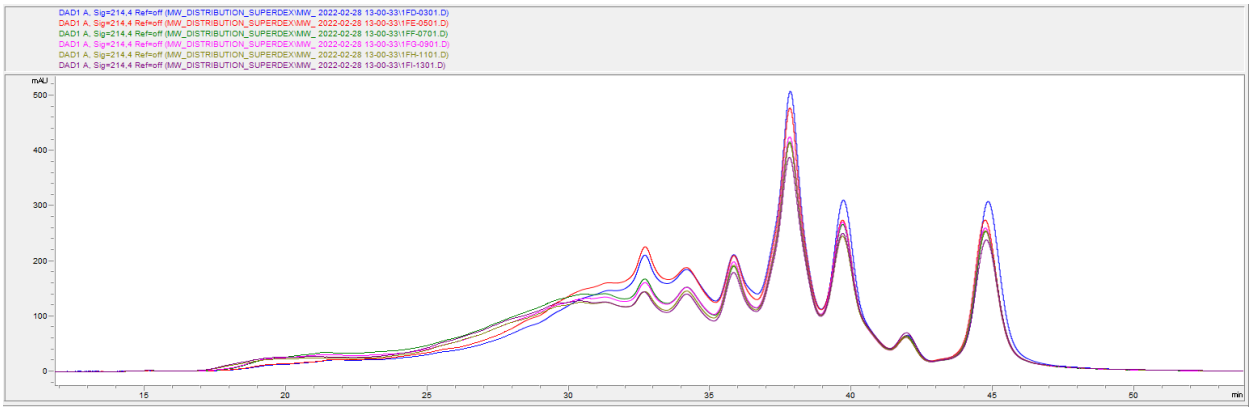


Figure 46: Chromatograms (mAU/min) from analysis of molecular weight distribution ($\bar{x}\pm$ SEM, n = 2) after membrane filtration of batch A (pearlside and krill) protein hydrolysates from non-enzymatic (C; control) and enzymatic (PB; papain + bromelain, Alc; alcalase) hydrolysis of whole raw materials. C1 (Blue), C2 (Red), PB1 (Green), PB2 (Pink), Alc1 (beige), Alc2 (Purple).

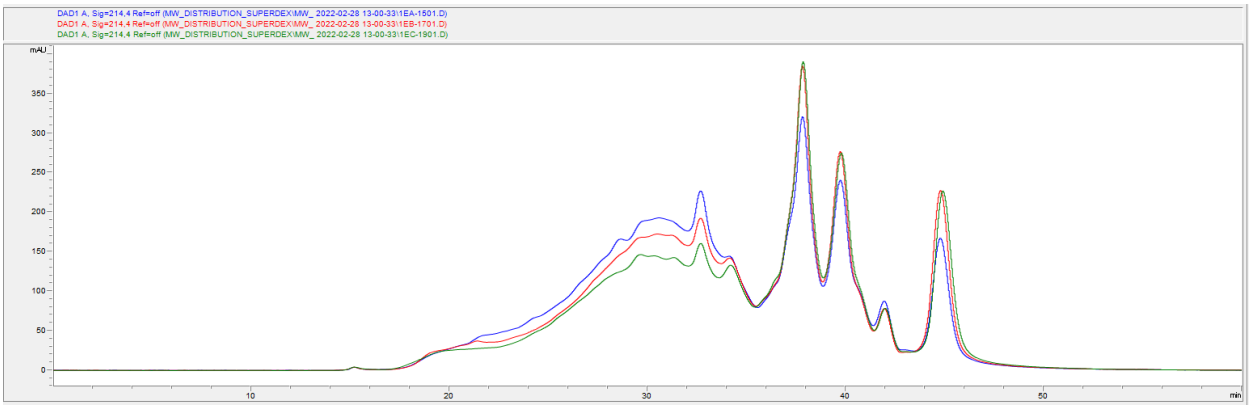


Figure 47: Chromatograms (mAU/min) from analysis of molecular weight distribution (n = 1) after membrane filtration of batch B (pearlside) protein hydrolysates from non-enzymatic (C; control) and enzymatic (PB; papain + bromelain, Alc; alcalase) hydrolysis of whole raw materials. C (Blue), PB (Red), Alc (Green).

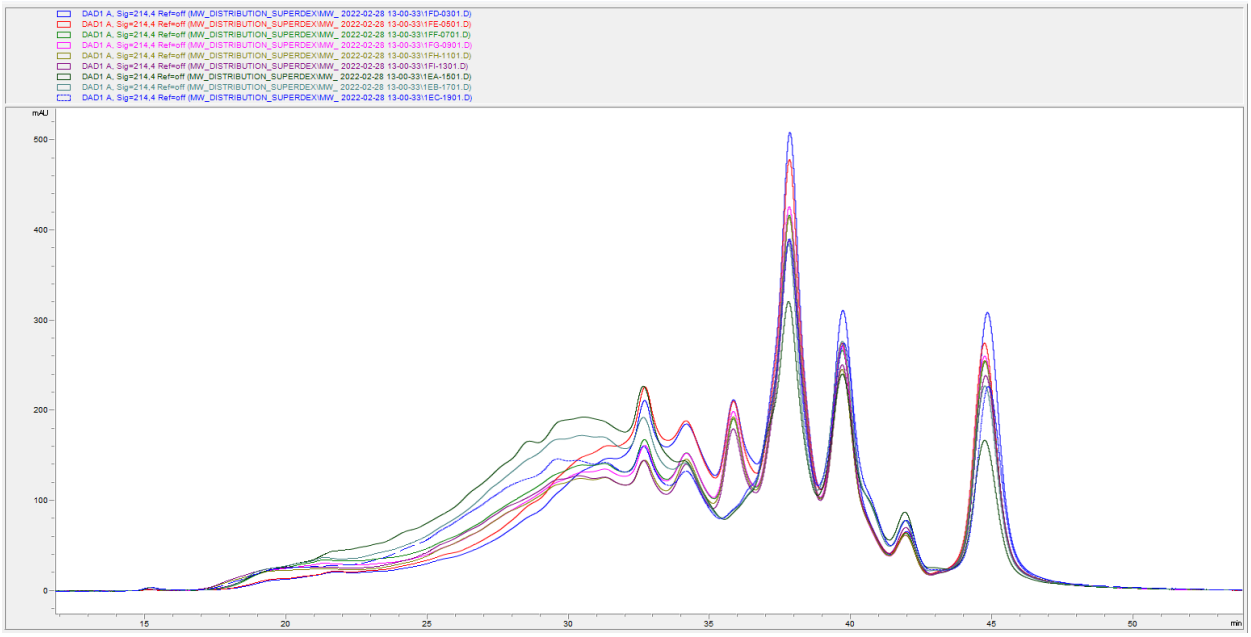


Figure 48: Comparism of chromatogram (mAU/min) of all the parallel of batch A to B Chromatograms (mAU/min) from analysis of molecular weight distribution ($\bar{x}\pm$ SEM, n = 4) for batch A, and ($\bar{x}\pm$ SEM, n = 2) for the batch B (C; control) and enzymatic (PB; papain + bromelain, Alc; alcalase). C1 (Blue), C2 (Red), PB1 (Green), PB2 (Pink), Alc1 (beige), Alc2 (Purple).

D ANOVA analysis of Antioxidant Assays

Figure 49: Batch A FRAP Anova and tukey post hoc error calculation. One way ANOVA was done using Tukey's multiple comparison test at $\alpha = 0.05$

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	726677.606	3	242225.869	72.555	<.001
Within Groups	13353.985	4	3338.496		
Total	740031.591	7			

		Subset for alpha = 0.05		
B	N	1	2	3
Raw	2	587.5650		
Control	2		1015.0450	
Alc	2		1153.3600	
PB	2			1421.1050
Sig.		1.000	.075	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Figure 50: Batch B FRAP Anova and tukey post hoc error calculation. One way ANOVA was done using Tukey's multiple comparison test at α = 0.05

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	82733.091	3	27577.697	13.284	.015
Within Groups	8303.868	4	2075.967		
Total	91036.959	7			

		Subset for alpha = 0.05	
B	N	1	2
Raw	2	274.4750	
Control	2		466.4450
Alc	2		491.9300
PB	2		543.1300
Sig.		1.000	.173

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Figure 51: Batch A ABTS Anova and tukey post hoc error calculation. One way ANOVA was done using Tukey's multiple comparison test at α = 0.05

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10717615.344	3	3572538.448	115.057	<.001
Within Groups	124200.116	4	31050.029		
Total	10841815.459	7			

		Subset for alpha = 0.05	
B	N	1	2
Raw	2	1286.4300	
Control	2		3683.0600
Alc	2		3948.6000
PB	2		4160.5850
Sig.		1.000	.057

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Figure 52: Batch B ABTS Anova and tukey post hoc error calculation. One way ANOVA was done using Tukey's multiple comparison test at α = 0.05

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	28207566.403	3	9402522.134	291.423	<.001
Within Groups	129056.504	4	32264.126		
Total	28336622.906	7			

		Subset for alpha = 0.05	
B	N	1	2
Raw	2	2320.7600	
Control	2		6490.3150
Alc	2		6635.9250
PB	2		6820.2350
Sig.		1.000	.146

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Figure 53: Batch A DPPH Anova and tukey post hoc error calculation. One way ANOVA was done using Tukey's multiple comparison test at α = 0.05

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4947690.475	3	1649230.158	290.372	<.001
Within Groups	22718.830	4	5679.708		
Total	4970409.305	7			

		Subset for alpha = 0.05		
B	N	1	2	3
Raw	2	57.7400		
Alc	2		1716.9900	
Control	2		1922.1500	1922.1500
PB	2			1947.3100
Sig.		1.000	.053	.755

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Figure 54: Batch B DPPH Anova and tukey post hoc error calculation. One way ANOVA was done using Tukey's multiple comparison test at α = 0.05

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	451185.433	3	150395.144	69.114	<.001
Within Groups	8704.198	4	2176.050		
Total	459889.631	7			

		Subset for alpha = 0.05		
B	N	1	2	3
Raw	2	81.9972		
Control	2		333.6620	
PB	2			647.7102
Alc	2			648.6866
Sig.		1.000	1.000	.984

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Figure 55: Batch A ORAC Anova and tukey post hoc error calculation. One way ANOVA was done using Tukey's multiple comparison test at α = 0.05

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	339388.689	3	113129.563	21.131	.006
Within Groups	21414.525	4	5353.631		
Total	360803.214	7			

		Subset for alpha = 0.05	
B	N	1	2
Raw	2	55.7650	
Alc	2		436.1200
Control	2		505.7050
PB	2		596.7900
Sig.		1.000	.098

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

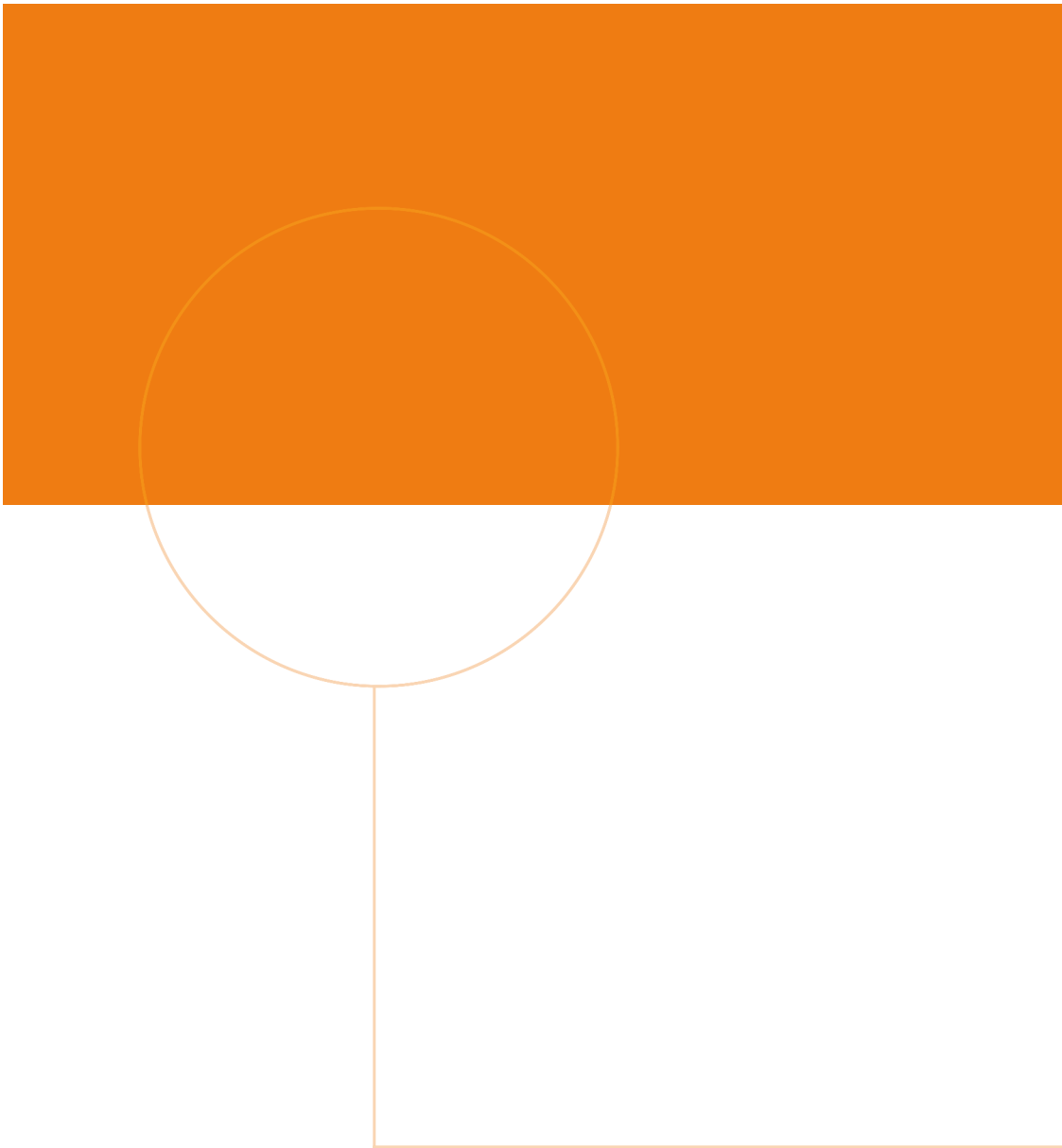
Figure 56: Batch B ORAC Anova and tukey post hoc error calculation. One way ANOVA was done using Tukey's multiple comparison test at $\alpha = 0.05$

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	517.647	3	172.549	15.470	.011
Within Groups	44.614	4	11.154		
Total	562.261	7			

		Subset for alpha = 0.05
B	N	1
Raw	4	36.8850
Alc	4	238.3700
Control	4	267.8375
PB	4	313.3900
Sig.		.166

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.



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