BIOLOGICALLY ACTIVE PROTEIN HYDROLYSATES FROM DOG FISH (*Squalus acanthias*) SKIN

By

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This thesis is dedicated to my beloved family

Abstract

In this study, dogfish (*Squalus acanthias*) skins were hydrolyzed with three different proteases, α-chymotrypsin, trypsin and papain, and evaluated for antioxidant, antimicrobial and enzyme inhibitory effects. The protein hydrolysates were produced by treating the skins with 1% enzyme for 4 h at 37˚C, followed by ultrafiltration using 10 kDa ultra filtration membranes. The antioxidant capacity of the hydrolysates based on their IC_{50} values for the α-Chymotrypsin treated protein hydrolysate (CPH) were calculated as 0.095 mg/mL, 0.116 mg/mL and 0.349 mg/mL, for superoxide anion scavenging, hydroxyl radical scavenging and metal ion chelating power, respectively; the corresponding values obtained with the trypsin treated protein hydrolysate (TPH) were 0.198 mg/mL, 0.186 mg/mL and 0.211 mg/mL; and the values for the papain treated protein hydrolysate (PPH) were 4.388 mg/mL, 0.096 mg/mL and 0.286 mg/ml, respectively. A reducing power of 0.5 was achieved with 0.940 mg/mL PPH, 1.083 mg/mL CPH and 3.70 mg/ml TPH. Initial antimicrobial testing with DH5α -*E.coli*, *Lactococcus lactis* and *Bacillus subtilis* with polymyxin B as positive control did not appear to have any antimicrobial activity by CPH, TPH and PPH. With the presence of these three hydrolysates survival of these probiotic bacteria (*Lactococcus lactis* and *Bacillus subtilis*) increased compared to their negative control. At 0.35mg/mL CPH produced 65% of inhibition against porcine pancreatic lipase, while TPH and PPH appeared to be enhancing the enzyme activity. At 40μg/mL, the inhibition (%) of α-chymotrypsin by the three hydrolysates was 32.5% (CPH), 16.4% (TPH) and 8.5% (PPH). The present study shows that enzymatically produced protein hydrolysates from dogfish skin have antioxidant and enzyme inhibitory properties as well as potential to improve probiotic bacteria survival. Further studies are needed to explore the antimicrobial effects, and to characterize the individual peptides in the hydrolysates to verify their potential as food processing aids.

Résumé

Dans cette étude, trois différents enzymes protéolytiques, soit α-chymotrypsine, trypsine, et papaine, ont été utilisés pour hydrolyser des échantillons de peau provenant d`aiguillat commun (*Squalus acanthias*). Les activités antioxidante, antimicrobienne et inhibitrice ont été mesurées sur les hydrolysats ainsi obtenus. Les réactions protéolytiques ont été conduites en traitant les échantillons de peau avec une concentration d`enzyme de 1 % durant 4 heures à 37^{o}C, suivi d`ultrafiltration sur membranes 10 kDa. La capacité antioxidante des hydrolysats par α-chymotrypsine (CPH), basé sur les valeurs $IC_{50}$ a été calculée comme suit : 0.095 mg/mL, 0.116 mg/mL et 0.349 mg/mL, respectivement envers l`anion superoxide, le radical hydroxyle, et pour le pouvoir de chélation minérale. Ces mêmes valeurs dans le cas des hydrolysats par trypsine (TPH) étaient respectivement de 0.198 mg/mL, 0.186 mg/mL et 0.211 mg/mL. Enfin, pour les hydrolysats par papaïne (PPH), des résultats correspondants de 4.388 mg/mL, 0.096 mg/mL et 0.286 mg/ml ont été mesurés. L`atteinte d`un pouvoir réducteur de 0.5 a exigé les concentrations suivantes de chaque hydrolysat: 0.940 mg/mL de PPH, 1.083 mg/mL de CPH et 3.70 mg/ml de TPH. Nos essais initiaux d`activité antimicrobienne DH5- *E.coli*, *Lactococcus lactis* et *Bacillus subtilis* avec polymyxine B comme témoin positif, n`ont mis aucune activité en évidence. En présence de chacun de ces trois hydrolysats, le taux de survie des bactéries probiotiques (*Lactococcus lactis* et *Bacillus subtilis*) a augmenté par rapport au témoin négatif. Pour une concentration de 0.35mg/mL le CPH a montré un taux d`inhibition de 65% envers la lipase pancréatique du porc, alors que TPH et PPH ont plutôt favorisé l`activité de cet enzyme. A 40µg/mL, le % d`inhibition de l`α-chymotrypsine observé pour ces trois hydrolysats était de 32.5% (CPH), 16.4% (TPH) et 8.5% (PPH). La présente étude a démontré que les hydrolysats protéiques obtenus à partir de la peau d`aiguillat par voie enzymatique possèdent des propriétés antioxidantes et inhibitrices, de même qu`une capactié à augmenter le taux de survie de certaines bactéries probiotiques. Des travaux supplémentaires sont requis afin de caractériser les peptides constitutifs de ces hydrolysats et également leurs effets antimicrobiens spécifiques; ceci permettra d`en valider le potentiel comme bioadditifs en transformation alimentaire.

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ACRONYMS

ROS	Reactive Oxygen Species
O^-_2	Superoxide Anion
OH	Hydroxyl Radical
H_2O_2	Hydrogen Peroxide
$^1O_2$	Singlet Oxygen
SOD	Superoxide Dismutase
BHT	Butylated Hydroxyl Toluene
BHA	Butylated Hydroxyl Anisole
PG	Propyl Gallate
TBHQ	Tertiary Butylhydroxy Quione
ACE	Angiotensin-Converting-Enzyme
DPPH	2,2-diphenyl-1-picrylhydrazyl
PUFA	Polyunsaturated Fatty Acid
HAT	Hydrogen Atom Transfer
SET	Single Electron Transfer
BDE	Bond dissociation energy
IP	Ionization Potential
ORAC	Oxygen Radical Absorbance Capacity
TEAC	Trolox Equivalence Antioxidant Capacity
FRAP	Ferric Ion Reducing Power
TBARS	Thiobarbituric Acid Reactive Substance
PV	Peroxide Value
AAPH	2,2′-Azo-Bis-2-Amidinopropanehydrochloride
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
PNP	4-Nitrophenylpalmitate
GSH	Glutathione
CPH	α-Chymotrypsin Treated Protein Hydrolysate
TPH	Trypsin Treated Protein Hydrolysate
PPH	Papain Treated Protein Hydrolysate

Chapter 1: INTRODUCTION

1.1. General Introduction:

Proteins are important biological compounds that exist naturally in all living organisms. They are made up of amino acids that are linked together by peptide bonds. Proteins, peptides and amino acids have beneficial effects on human health and nutrition. Generally dietary proteins are useful for maintenance of proper growth and functioning. They also possess many beneficial biological functions. These characteristics are mainly attributed to certain peptide sequences and conformations. These biologically active peptides are obtained by hydrolysis of protein into protein hydrolysates. The protein hydrolysates are observed to be more active than when they are in their parent protein. Biologically active protein hydrolysates are specific in their action thus demonstrates a positive impact on body functions and may ultimately influence health (Kitts & Weiler, 2003).

Some bioactive protein hydrolysates are very resistant to the action of peptidases (Kitts & Weiler, 2003). Thus their functional and biological properties are not affected during the gastrointestinal digestion; hence accomplish their targeted task successfully. Protein hydrolysates have influenced recent scientific research interest as they tend to decrease the risk of chronic diseases and also help in promoting a healthy life (Hannu & Anne, 2003). Due to the small size and free exposure of their functional groups than when they are in their parental protein, bioactive protein hydrolysates are more effective in their targeted tasks (Hannu & Anne, 2003).

Biologically active protein hydrolysates have many physiological functions that impact the cardiovascular system, nervous system, gastrointestinal system and immune system by expressing different properties like anti-hypertensive, antioxidant, anti-thrombotic, hypocholestrolemic, opioid, mineral-binding, appetite suppression, anti-microbial, enzyme inhibitory, immunomodulatory and cytomodulatory. There are many synthetic drugs which have same effects as biologically active protein hydrolysates. But the long term intake of chemically synthesized drugs causes many adverse side effects to human health.

1.2. Sources of bioactive protein hydrolysates:

Biologically active protein hydrolysates have been isolated from bacterial, fungal, plant and animal sources. For example bioactive protein hydrolysates have been produced from milk, cereals, egg, meat and fish protein and shown to have potential of enhancing human health. Research on bioactive protein hydrolysates was initiated using milk proteins. Milk derived bioactive protein hydrolysates act as prominent candidates for various health promoting function pertaining to the heart, bone and also the immune system. Hydrolysates and peptides from whey protein have been linked with enhancing natural immune response and to reduce the risk of cancer, decrease the severity of degeneration of muscle tissue which is related to liver disease, and also lower susceptibility to diarrhea (Hannu & Anne, 2003). Studies on bioactive protein hydrolysates and peptides have provided ample data attesting to their potential for applications in the food, nutraceutical and pharmaceutical fields. For example, lactoferricine is an antimicrobial peptide obtained by enzymatic degradation of the milk protein lactoferrin, with pepsin (Tomita, Wakabayashi, Yamauchi, Teraguchi, & Hayasawa, 2002)**,** and certain bacteriocins are antimicrobial peptides derived from milk proteins via microbial fermentation by LAB (Fitzgerald & Murray, 2006). Glycomacropeptide [GMP] is a casein peptide that plays a significant role in appetite suppression (Bohdan L. Luhovyy, Tina Akhavan, & Anderson, 2007). GMP is involved in stimulating the production of an intestinal hormone called cholecystokinin which induces the feeling of satiety. Soybean protein is another important source of bioactive peptides. There are several studies with soy protein which demonstrate that peptides from soy protein have antimicrobial, antioxidant and antihypertensive properties (Cai, Gu, Li, Ma, Dong, Liu, et al., 2012; W. Wang & De Mejia, 2005). Protein hydrolysates play another role in food processing by acting as a flavour enhancer. The digested protein fragments with sensory properties also act as pharmaceutical and nutritive products (Moure, Dominguez, & Parajo, 2005).

Fish skins from commercial processing are mostly discarded by the fish industries which results to pollute the environment; otherwise they must be disposed of at high cost to the companies. A fraction of fish processing by-products are converted to fish oil, or low value products such as fertilizer and animal feed (Choudhury & Bublitz, 1996; Yang, Ke,

Hong, Zeng, & Cao, 2011). However, studies show that these by-products may potentially be transformed into high value-added products such as biochemicals (e.g., enzymes), cosmetics and pharmaceutical products (Aneiros & Garateix, 2004). In order to convince the industries for the development of value-added products such as bioactive protein hydrolysates or peptides from food processing by-products, the processing cost must be feasible compared to discarding or using them as feed and fertilizers (Kristinsson & Rasco, 2000b; Meyers, 1986).

Dogfish is used in Canada, Europe, New Zealand, USA, China and Germany as food due to its high nutritious meat (Stefania Vannuccini, 1999). Consumption of dogfish in European and Asian countries is high than Canada, thus they are abundantly available in North America. It belongs to Squalidae family with a genus and species name of *Squalus acanthias*. Apart from being a food, dogfish meat and waste like fish skin are used as animal feed and fertilizers. Squalime which is an aminosterol extracted from liver tissue of dogfish acts as a broad spectrum antimicrobial agent (Karen S. Moore, Suzanne Wehrl, Henirich Roder, Mark Rogers, John N. Forrest, Donald Mccrimmon, et al., 1993). Dogfish skin does not contain scales as Osteichthye fishes. Because of prior studies showing compound(s) with antimicrobial agent in dogfish and also because the skins are mostly discarded as waste dogfish skin was studied to verify its protein hydrolysates and peptides for bioactivities such as antioxidant, antimicrobial and enzyme inhibitory effects.

1.3. Production of Bioactive protein hydrolysates:

Bioactive protein hydrolysates are produced naturally from the breakdown of dietary protein during gastrointestinal digestion and absorbed in the intestinal epithelial tract (Young & Mine, 2009). The stomach enzyme, pepsin**,** catalyzes the hydrolysis of proteins into larger oligopeptides that are subsequently cleaved into shorter chain peptides by intestinal digestive protease such as chymotrypsin and trypsin. Dietary proteins undergo further changes due to variation in buffering capacities by a variety of brush border membranes and intracellular peptidase that can further hydrolyze the tryptic and chymotryptic peptides into even shorter chain peptides and free amino acids.

In addition to the gastrointestinal digestion of protein into peptides (with or without bioactivities), the latter compounds may also be produced by *in vitro* methods chemical hydrolysis, enzyme hydrolysis or microbial fermentation (J. K. Lee, Jeon, Kim, & Byun, 2012). Chemical hydrolysis includes acid hydrolysis, alkaline hydrolysis and solvent extraction. Chemical hydrolysis is simple and less expensive compared to enzyme hydrolysis, but this method of extraction is not specific and not easy to control. Thus the production of peptide could differ from batch to batch (Lordan, Ross, & Stanton, 2011). Bioactive peptides are also produced by microbial fermentation (Pasupuleti & Braun, 2010). Enzyme hydrolysis involves hydrolysis by digestive enzymes, enzymes that may be derived from microorganism, plants or animals. Enzymatic hydrolysis has the advantage of mimicking gastrointestinal digestion of protein, and there have been extensive studies carried out that were based on enzyme assisted production of biologically active protein hydrolysates. The advantages with the use of enzymes include its high specificity, ease of control consistent product from batch to batch, and effectiveness under mild conditions pH and temperature. The most commonly used proteolytic enzymes that have been used to produce bioactive peptides include papain, pepsin, trypsin, chymotrypsin and alcalase. Chymotrypsin, trypsin and papain are generally used for meat tenderisation in food industry (Lantto, Kruus, Puolanne, Honkapaa, Roininen, & Buchert, 2010). Papain is extensively used for making higher level nutriments, cereals and beverages make soluble protein products and breakfast, gelatin stabilization, health food, dry fermentation and food ripening (Zbigniew, Franciszek, & Wiestaw, 2007). Trypsin and Chymotrypsin are used in food processing as a baking enzyme to improve the workability of dough, to control aroma formation in cheese and milk products; in the extraction of seasonings and flavourings from vegetable or animal proteins and in the manufacture of sauces; to improve the texture of fish products; during cold stabilization of beer (Arason, 2002).

Following the production of the hydrolysates by enzymatic hydrolysis, the enzymes used for hydrolysis must be inactivated in the reaction mixture to avoid potential negative effects from excessive hydrolysis. Traditionally treatment by pH and temperature adjustment is done to inactivate the enzyme. Alternatively separation of enzyme from the reaction mixture can be carried out using filtration and centrifugation processes

(Bourseau, Vandanjon, Jaouen, Chaplain-Derouiniot, Massé, Guérard, et al., 2009). Ultra filtration is the best suited method for the separation of low molecular weight bioactive peptides from high molecular weight hydrolyzing proteolytic enzyme and nonhydrolysed proteins (Bourseau, et al., 2009).

The peptides in the hydrolysates are further purified by various chromatographic and electrophoretic techniques to verify bioactivities, sequence, size and other structural and functional properties in order to justify their use for various purposes.

1.4. Research objectives:

a. To investigate enzymatic hydrolysis of dogfish skin using three different proteolytic enzymes, namely, α-chymotrypsin, trypsin and papain.

b. To characterize the hydrolysates for antioxidant, antimicrobial and enzyme inhibitory properties.

Chapter 2: LITERATURE REVIEW

2.1. What are Antioxidants?

Antioxidants are compounds that protect other compounds or molecules from oxidative damage. In food and biological systems, antioxidants protect against oxidative damage by controlling the scavenging effects of reactive species such as: free radicals (e.g., hydroxyl radicals, alkoxy and peroxy radicals), reactive oxygen species (e.g., singlet oxygen, superoxide radical), peroxides, organic hydroperoxides, peroxynitrite, hypoclorous acid and others (Huang, Boxin, & Prior, 2005). In food, antioxidants delay or prevent autooxidation of food components like lipids to curtail rancidity and spoilage (Mielnik, Aaby, & Skrede, 2003) . They can also protect against oxidation of food proteins to maintain protein functionality and on specific qualities as in muscle foods (Lund, Heinonen, Baron, & Estévez, 2011).

2.1.1. General view of Antioxidant:

2.1.1.1. Role of Antioxidants in human health:

Oxidation is an important process in living organism, even though it involves in production of free radicals which tend to be the main cause for many diseases and pathogenesis specially in human (Ren, Zhao, Shi, Wang, Jiang, Cui, et al., 2008). Free radicals are species which have one or multiple unpaired electrons that are formed during normal metabolism, such as during aerobic respiration, lipid degradation, also during stress and inflammatory processes (Gülçin, Büyükokuroğlu, Oktay, & Küfrevioğlu, 2003; D. Wang, Wang, Zhu, Zhu, Chen, Zou, et al., 2008). Reactive oxygen species [ROS] like superoxide anion [O^{-}_{2}], hydroxyl radical [OH], hydrogen peroxide [H_2O_2] and singlet oxygen [$^{1}O_2$] are few free radicals. When an excess of free radicals is formed, they can overwhelm protective enzymes like superoxide dismutase, catalase and peroxidase, and cause destructive and lethal cellular effects [e.g. apoptosis] by oxidizing membrane lipids, cellular proteins, DNA and enzymes, thus shutting down cellular respiration (Pihlanto, 2006). Oxidation and antioxidation balance in living system helps in maintaining healthy

tissues and organs during normal physiological process. When there is an imbalance between the oxidation and antioxidation processes, excess free radicals accumulate that can cause oxidative damage to damage biological molecules and tissues to produce many harmful diseases like cancer, stroke, diabetes, atherosclerosis, rheumatoid arthritis. Many studies on Alzheimer's patients show that there is an increased oxidative damage to all the major classes of biomolecules in their brains (J. Wang, Xiong, Xie, Markesbery, & Lovell, 2005). Also several studies on various cancers show that it is probably a consequence of oxidative DNA-damage (Cooke, Evans, Dizdaroglu, & Lunec, 2003). Under normal conditions the oxidation process is controlled by enzymatic [catalase, superoxide dismutase and glutathione peroxidase] and non-enzymatic antioxidants [selenium, α- tocopherol and vitamin C] (Rahman, 2007).

2.1.1.2. Role of Antioxidants in food:

Oxidation is a phenomenon in food industry especially in terms of food quality and food safety. It is one of the major causes of food deterioration. The important deteriorations in food are colour change, microbial growth and lipid oxidation. Oxidation affects lipids, carbohydrates and protein in food, where lipid oxidation causes rancidity as well as flavor and colour changes, protein oxidation in protein functionality and texture modification in food. Other changes caused by oxidation of food components include loss of nutritional value, loss of enzyme activity, and formation of toxic metabolites and carcinogenic substances (Pihlanto, 2006). These adverse effects will ultimately lead to reduction of shelf life. There are several methods which are employed in food industry for preventing lipid oxidation, but the addition of antioxidant is considered to be the most effective, convenient and economical (Shahidi & Zhong, 2011).

2.1.1.3 Exogenous and Endogenous source of ROS:

In general ROS are formed exogenously or endogenously. Exogenous sources of ROS are generated by cigarette smoking, alcohol consumption, radiation, organic solvents, anaesthetic gases, hypertoxic environments and pesticides (Riley, 1994), whereas endogenous ROS production is via processes such as activation of phagocytic cells in peroxisomes where fatty acids are degraded, and by auto-oxidation of various molecules,

(Halliwell, Gutteridge, & Cross, 1992). This oxidative stress can be controlled by antioxidant defence mechanism that includes endogenous enzymes like superoxide dismutase [SOD], glutathione peroxidase and catalase, natural antioxidants [α-tocopherol, β-carotene and ascorbic acid] and dietary antioxidants (Halliwell, Gutteridge, & Cross, 1992). Antioxidant helps in preventing or to slow down oxidative damage inside our body.

2.1.2. Sources of antioxidants:

There are two main sources of antioxidants that are used to protect food from oxidative damage. The antioxidants are derived from synthetic and natural sources.

2.1.2.1. Synthetic sources:

Synthetic antioxidants like BHT [butylated hydroxyl toluene], BHA [butylated hydroxyl anisole], PG [propyl gallate], *2-6*-di-tert-butyl-*4*-hydroxymethyl-phenoland TBHQ [tertiary butylhydroxy quione] are widely used due to their high effects and cheaper value compared to natural antioxidants (Yanishlieva, 2001).

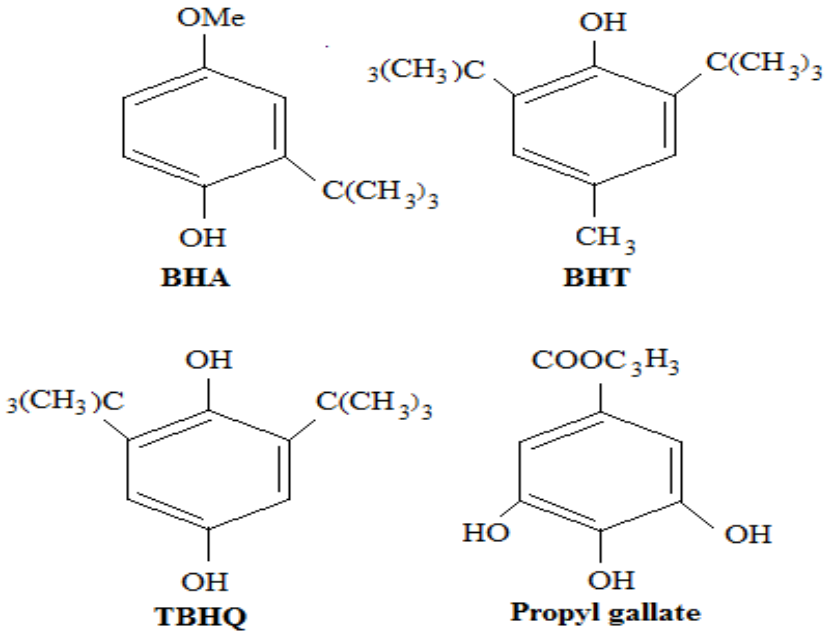

Figure: 2.a. Structure of synthetic antioxidants (Yanishlieva, 2001)

But scientific investigations suggest potential adverse toxic and carcinogenic effects due to the consumption of high amounts of synthetic antioxidants, thus the use of synthetic antioxidants in foodstuffs are restricted or highly regulated in many countries (Miková, 2001).

According to a National Toxicology Program Report, propyl gallate [3,4,5-trihydroxybenzoic acid propyl ester] induced preputial gland tumours, islet cell tumours of pancreas and pheochromocytomas of adrenal glands in experimental animals ("Final Report on the Amended Safety Assessment of Propyl Gallate," 2007). The toxic effects of PG to aquatic organisms were investigated and PG cytotoxicity was reported to be dependent on glutathione levels while general antioxidant levels did not cause any change in the toxic level of PG. Thus, it was suggested that it has been classified under toxic substance for aquatic organism.

The toxicology of the food preservatives like butylhydroxyanisole [BHA] and butylhydroxytoluene [BHT] indicates that exposure to high doses of these compounds induces impairment of blood clotting in experimental animals (Kahl & Kappus, 1993). BHT causes specific toxic effects to the lung (Kahl & Kappus, 1993). BHA was also reported to induce tumours in the fore stomach of rats when N-methyl-N'-nitro-N-nitroguidine, N-methlnitrosourea or N-dibuttynitrosamine was used as initiator, whereas BHT induced liver tumours in long-term experiments (Kahl & Kappus, 1993).

TBHQ is permitted for food use by the FDA and the USDA at less than 0.02% and 0.01%, respectively (Shahidi & Zhong, 2005). TBHQ by itself is not considered as a carcinogen, but studies using high doses with rats or mice, produced a metabolite in urinary tract that was found to be toxic to kidney and bladder.

2.1.2.2. Natural sources:

Several studies have shown a variety of natural compounds to have antioxidant properties. Examples of these include tocopherols, vitamin C, flavonoids, phytochemicals and certain peptides (e.g., glutathione) and sulphur containing and aromatic amino acids such as cysteine, methionine, tryptophan and proline, (Jun, Park, Jung, & Kim, 2004; Marcuse, 1960).

Plant foods like soy beans and pulse crops serve as excellent sources of bioactive protein hydrolysates and peptides with antioxidant activities (Gibbs, Zougman, Masse, & Mulligan, 2004; Roy, Boye, & Simpson, 2010). Peptide hydrolysates from milk, egg, meat and fish have all been shown to have antioxidant peptides.

2.1.2.2.1. Animal-derived antioxidant protein hydrolysates:

Milk naturally contains many macro and micro nutrients. Protein hydrolysates from milk proteins, casein and whey protein have been reported to display several biological activities like antiviral, antimicrobial, antioxidant, ACE inhibitor and anticarcinogenic activities. Based on this, casein-derived peptides are being used as food and pharmaceutical supplements (Phelan, Aherne, FitzGerald, & O'Brien, 2009). For example caseinophosphopeptides obtained from milk casein are being produced on industrial scale for applications in functional food and pharmaceutical products. Caseinophosphopeptides can form soluble organophosphate salts and may function as carriers for different minerals, especially calcium. From the study by Rival, Boeriu, and Wichers (2000), it is understood that casein derived peptides exhibit chelating activity, inhibition of lipid peroxidation by lipoxygenase and inactivation of free radicals.

Eggs are excellent source of protein, lipids, vitamins and minerals. Both egg white and egg yolk derived peptides have been studied for antioxidant properties like scavenging superoxide, hydroxyl and peroxyl radicals, and inhibiting lipid oxidation (B & R, 2011). Antioxidative capacity of hydrolysates of hen egg ovalabumin hydrolysates was studied by Xu M, Shangguan X, Wang W, and J. (2007) showed the inhibition capacity of hydrolysates against superoxide anion and $HO^{\cdot}$ were more than 45% and 56% respectively at the concentration 5 mg/mL.

2.1.2.2.2. Plant-derived Antioxidant protein hydrolysates:

Fruits, vegetables, cereals, grains, oil seeds, and teas serve as good sources of antioxidants. Among the plant derived antioxidants, soy protein hydrolysates have demonstrated number of antioxidant properties like scavenging free radicals, metal-ion chelating, oxygen quencher, and also inhibited compounds that initiates lipid oxidation. A

study by Chen, Muramoto, Yamauchi, and Nokihara (1996) reported that soy peptides demonstrated antioxidant activity against the peroxidation of linoleic acid in an aqueous system. A peptide sequence of Leu-Leu-Pro-His-His from soy protein exhibited strong radical scavenging activity (Chen, Muramoto, Yamauchi, & Nokihara, 1996).

Potato protein hydrolysates [PPH] was studied by Y. Cheng, Xiong, and Chen (2010), reported that antioxidative PPH prepared by alcalase was highly inhibitory of lipid oxidation in a soybean oil-in-water emulsion and attributed the behaviour to radical scavenging ability of the protein hydrolysates.

Plant derived antioxidant protein hydrolsates do not occur only in fruits, vegetables and leaves, but also in flowers (Megías, Pedroche, Yust, Girón-Calle, Alaiz, Millán, et al., 2007). Megías, et al. (2007) demonstrated sunflower protein hydrolysates had copper ion chelating activity.

2.1.2.2.3. Antioxidant protein hydrolysates from Marine sources:

Bioactive protein hydrolysates have been characterized from marine sources and shown to exhibit beneficial effects like scavenging free radicals or reactive oxygen species and preventing oxidative damage by interrupting the radical chain reaction in lipids (Mendis, Rajapakse, Byun, & Kim, 2005; Qian, Jung, Byun, & Kim, 2008; Rajapakse, Mendis, Byun, & Kim, 2005; Ranathunga, Rajapakse, & Kim, 2006).

Various studies suggest that fish protein hydrolysates are beneficial due to their high content of bioactive molecules that are directly available in digested form (Harnedy & FitzGerald, 2012).

Collagen and gelatin hydrolysates have also been shown to have bioactive peptides which have antioxidant, antihypertensive and antimicrobial activities. A study by Kim, Kim, Byun, Nam, Joo, and Shahidi (2001) indicated that peptides isolated from Alaskan pollack using alcalase, pronase E, and collagenase exhibited antioxidant activity and could protect rat liver cells from oxidative damage by organic hydroperoxide t-BHP. Similarly, Alemán, Pérez-Santín, Bordenave-Juchereau, Arnaudin, Gómez-Guillén, and Montero (2011) observed that protein hydrolysates from squid skin and tuna skin gelatins

had effective reducing power and radical scavenging activity. There are several studies done to verify antioxidant properties of seaweed (Vijayabaskar & Shiyamala, 2012). Seaweed pipefish [*Syngnathus schlageli*] hydrolysate is being used as a medicine in China. The hydrolysate was prepared using alcalase, neutrase, papain, pronase E and trypsin and had two novel peptides with molecular weights of 809.97 and 807.9 Da, with amino acid sequences of SVMPVVA and QLGNLGV. These two peptides exhibited DPPH, superoxide, hydroxyl and alkoxyl free radicals scavenging activities. Among the free radicals the peptides showed highest inhibition of hydroxyl radical.

Peptide sequences [Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu, Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg] from jumbo squid display high rate of inhibition of lipid oxidation compared with α-tocopherol and similar to BHT (Mendis, Rajapakse, Byun, & Kim, 2005). The sequence of a peptide derived from oyster [Leu-Lys-Glu-Glu-Leu-Glu-Asp-Leu-Glu-Lys-Glu-Glu] was shown to exhibit high antioxidant activity against peroxidation of polyunsaturated fatty acids than α-tocopherol (Qian, Jung, Byun, & Kim, 2008). Byun, Lee, Park, Jeon, and Kim (2009) studied protein hydrolysate from rotifer, *Brachionus rotundiformis* produced using enzymes like alcalase, trypsin, pepsin, neutrase, α-chymotrypsin and papain. The peptides were verified for DPPH radical scavenging activity, and shown that the EC_{50} values of 189.8 and 167.7μM. The antioxidant peptides were identified as Leu-Leu-Gly-Pro-Gly-Leu-Thr-Asp-His-Ala [1076 Da] and Asp-Leu-Gly-Leu-Gly-Leu-Pro-Gly-Ala-His [1033Da] by Q-TOF ESI mass spectroscopy method (Byun, Lee, Park, Jeon, & Kim, 2009). The sequence of the peptides from the protein hydrolysates from the skins of horse mackerel [Asn-His-Arg-Tyr-Asp-Arg, 856 Da] and croaker [Gly-Asn-Arg-Gly-Phe-Ala-Cys-Arg-His-Ala ,1101.5 Da] exhibited DPPH radical scavenging activity, with both peptides showing higher activity against polyunsaturated fatty acid [PUFA] peroxidation than the natural antioxidant α-tocopherol (Sampath Kumar, Nazeer, & Jaiganesh, 2012). The antioxidant activity against PUFA peroxidation was attributed to specific scavenging of radicals formed during peroxidation, scavenging of oxygen-containing compounds, or metal chelating ability (Sampath Kumar, Nazeer, & Jaiganesh, 2012).

Several studies suggest that marine derived peptides have higher antioxidant activity than α-tocopherol (Jun, Park, Jung, & Kim, 2004; Rajapakse, Mendis, Byun, & Kim, 2005). However, using these marine derived antioxidant peptides in food has some drawback such as eliciting bitter taste in the products (Kristinsson & Rasco, 2000a). A study by Shahidi, Han, and Synowiecki (1995) found that treatment of the fish derived antioxidant peptide with activated carbon could eliminate the bitter taste.

Bigeye tuna head was studied for antioxidant properties. The hydrolysis was effected with alcalase enzyme and the isolated peptides were investigated for antioxidant properties, and found to have IC_{50} values for 1,1-diphenyl-2-pycrylhydrazyl [DPPH], superoxide and hydroxyl radicals as 1.34, 1.20 and 2.84 mg/mL, respectively; and its reducing power was 0.948 at 12.5 mg/mL.

2.1.3. Reaction Mechanisms of Antioxidants:

Antioxidants perform in two different mechanisms, i.e., by hydrogen atom transfer [HAT] and single electron transfer [SET] mechanisms. Though their inactivation mechanism, kinetics and potential for side reaction are different, the end result is the similar. Single electron transfer and Hydrogen atom transfer reactions may occur in parallel. There are also other factors that play a role in measuring the ability of antioxidant in a given system, like antioxidant structure and properties, solubility and partition coefficient, and system solvent (Wright, Johnson, & Dilabio, 2001). Bond dissociation energy [BDE] and ionization potential [IP] (Bulet, Stöcklin, & Menin, 2004) are two major factors that determine the mechanism and efficacy of antioxidants (Prior, Wu, & Schaich, 2005 ; Wright, Johnson, & Dilabio, 2001).

The HAT mechanism evaluates the quenching efficiency of an antioxidant over free radicals by hydrogen donation [AH = any H donor]

$$\mathbf{X^{\cdot} + AH \longrightarrow XH + A^{\cdot}}$$

$X^{\bullet}$- Free radical, AH- any presumed antioxidant compound able to act as hydrogen atom donor.

HAT based assay in general work with a synthetic free radical generator, an oxidizable probe and an antioxidant. With reference to the study by Wright, Johnson, and Dilabio (2001) the relative reactivity in HAT-based methods is measured by the BDE of the H-donating group in the potential antioxidant, dominating for compounds with ΔBDE of ~ −10 kcal/mol and ionization potential [ΔIP] of < −36 kcal/mol. HAT reactions are usually rapid, and generally over within seconds to minutes so antioxidant reactivity or capacity measurements are based on competition kinetics. HAT reactions are independent of solvent and pH effects. The presence of reducing agents, like metal ions, usually is a complication in HAT based assay and can lead to erroneously high apparent reactivity (Prior, Wu, & Schaich, 2005).

The SET-based mechanism involves measurements of the ability of a potential antioxidant to transfer one electron to reduce any compound, like metal ions, carbonyls, and radicals. In this method the cation is first formed followed by rapid and reversible deprotonation in solution, according to

$$\mathbf{X}^{\cdot} + \mathbf{AH} \longrightarrow \mathbf{XH} + \mathbf{A}^{\cdot +} \quad \text{(Electron transfer)}$$

$$\mathbf{AH}^{\cdot +} + \mathbf{H_2O} \longleftrightarrow \mathbf{A}^{\cdot} + \mathbf{H_3O}^{+} \text{ (Deprotonation equilibrium)}$$

$$\mathbf{X}^{\cdot} + \mathbf{H_3O}^{+} \longrightarrow \mathbf{XH} + \mathbf{H_2O} \text{ (Hydroperoxide formation)}$$

SET and HAT mechanisms generally occur together in all samples, with the balance determined by antioxidant structure and pH (Prior, Wu, & Schaich, 2005). Relative reactivity in SET methods is based primarily on deprotonation and IP of the reactive functional group, so SET reactions are strongly pH and solvent dependent due to solvent stabilization of the charged species (Prior, Wu, & Schaich, 2005). In general, with increase in pH, IP values decrease, which results in increased electron-donating capacity with deprotonation (Prior, Wu, & Schaich, 2005). The antioxidant compounds with a ΔIP of > −45 kcal/mol have SET mechanism (Prior, Wu, & Schaich, 2005).

SET based antioxidant capacity is calculated in terms of percent decrease in product rather than kinetics as their reactions are usually slow and can require long times to reach completion (Se-Kwon, Isuru, Eun, Yasuki, Yasushi, & Kenji, 2011). The SET methods are sensitive to the presence of trace components and contaminants [particularly metal

ions] and their interference can account for high variability and poor reproducibility and consistency of results (Se-Kwon, Isuru, Eun, Yasuki, Yasushi, & Kenji, 2011).

HAT based assays include oxygen radical absorbance capacity [ORAC] assay and total trapping antioxidant parameter assay. SET based assays quantify the capacity of an antioxidant to reduce an antioxidant, which results in colour change. These assays measure the radical scavenging power of an antioxidant. SET based assays include trolox equivalence antioxidant capacity assay [TEAC], the ferric ion reducing power assay [FRAP], total antioxidant potential assay, 2,2-diphenyl-1-picryl-hydrazyl DPPH assay, hydroxyl radical scavenging activity, superoxide radical scavenging assay and peroxyl radical scavenging assay. Other assays for antioxidant activity include measurements of thiobarbituric acid reactive substance [TBARS] assay and peroxide value [PV] assays (Prior, Wu, & Schaich, 2005; Se-Kwon, Isuru, Eun, Yasuki, Yasushi, & Kenji, 2011).

2.1.4. Methods to determine antioxidant activity:

There are several methods available for measuring antioxidant activity (Zulueta, Esteve, & Frígola, 2009). The most commonly used methods employed to study antioxidant activity of protein hydrolysates are described below:

2.1.4.1. DPPH Free Radical Scavenging Activity:

$$\mathbf{DPPH^{*} + AH \longrightarrow DPPH\text{-}H + A^{*}}$$

DPPH* radical is a stable organic nitrogen radical (Prior, Wu, & Schaich, 2005). The principle of this spectrophotometric procedure is based on the scavenging ability of specific antioxidant [AH] with a stable free radical 2, 2-diphenyl-1-picryl-hydrazyl DPPH* (Buijnsters, Bicanic, Chirtoc, Nicoli, & Min-Kuo, 2001). The reaction is light sensitive, thus incubated in dark and measured at 517 nm. A lower absorbance represents a higher DPPH scavenging activity.

2.1.4.2. Hydroxyl Radical Scavenging Antioxidant Activity:

$$\mathbf{Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\cdot}OH + OH^{\cdot}}$$

The above reaction describes the role of Fe^{2+} in production of $^{\bullet}OH$ by the Fenton reaction (Bektaşoğlu, Esin Çelik, Özyürek, Güçlü, & Apak, 2006). The chemistry

of the assay is [Fe^{3+}]-EDTA complex reacts with H_2O_2 thus $^{\bullet}OH$ radicals are generated. $^{\bullet}OH$ radicals cause site specific degradation to the deoxyribose probe molecule and form smaller molecular fragment, which produce malonialdehyde a pink chromogenic compound when heated with thiobarbituric acid [TBA]. Addition of TCA stops further formation of maldondialdehyde as a result of reduced pH. Amount of Malonialdehyde formed is measured at 532nm. A hydroxyl radical scavenger added to the medium reduces Fe^{3+} to Fe^{2+} which initiates Fenton reaction to form hydroxyl radical in presence of H_2O_2 (Bektaşoğlu, Esin Çelik, Özyürek, Güçlü, & Apak, 2006). Thus added hydroxyl radical scavenger reduces the degradation of deoxyribose sugar which results in diminishing of pink color formation , enabling the calculation of second-order rate constants of $^{\bullet}OH$ scavenging. A decrease in the absorbance of the reaction mixture indicates decreased oxidation of deoxyribose.

2.1.4.3. Superoxide Anion Scavenging Assay:

Pyrogallol [1,2,3-benzenetriol] has long been known to autoxidize rapidly, especially in alkaline solution and the reaction has been employed to measure the removal of oxygen from gases (Marklund & Marklund, 1974) . Scavenging activity of the antioxidant was calculated as the inhibition rate of pyrogallol auto-oxidation at 325 nm.

2.1.4.4. Reducing Power Assay:

This assay is based on the reduction of ferric iron to ferrous ion result in the formation of Perl Prussian blue color, that is at 700nm (Singh & Rajini, 2004). Increased absorbance of the reaction mixture is an index increased reducing power.

2.1.4.5.. Oxygen Radical Absorbance Capacity Assay:

The ORAC assay measures the ability of antioxidant to inhibit peroxyl radicals formations which are induced during oxidation by breaking radical chain reaction via the transfer of H atoms. Generally ORAC method works with a fluorescent

probe which reduces in fluorescent colour due to reaction with free radicals (Prior, Wu, & Schaich, 2005). β-phycoerythirin is a water-soluble protein product isolated from *Porphyridium cruentum* with fluroscent colour generally used in ORAC method. β-phycoerythirin is sensitive towards reactive oxygen species, thus with the action of free radicals over the fluorescent probe it reduces the colour. In presence of antioxidant the degree of reduction of colour is reduced. However, due to lack of constant reproducibility in β-phycoerythirin and ability of losing fluorescent color when exposed to light, this probe is less recommend for ORAC assay. Thus it is recommended making use of other fluorescent probes that are stable during exposure of light, and have high molar extension coefficient and quantum yield (Prior, Wu, & Schaich, 2005). Probes like fluorescein and 6-carboxyfluorescein are highly suggested. The fluorescein and its derivatives are excited at 490 nm and are read at 514 nm (Prior, Wu, & Schaich, 2005).

2.1.4.6. Total Reactive Antioxidant Potential Assay:

$$\mathbf{ROO\cdot + AH \longrightarrow ROOH + A\cdot}$$

The principle of TRAP assay is similar to the ORAC assay. It measures the capacity of antioxidants to inhibit peroxyl radicals. The assay includes a luminol probe like 2,2′-Azo-bis-2-amidinopropanehydrochloride (AAPH) or (ABPH). Peroxyl radicals are generated by thermal decomposition of AAPH at a known and constant rate (Prior, Wu, & Schaich, 2005). During the oxidation of AAPH it produces luminol radicals which emit light. Thus the principle of the assay is to measure the reduction or inhibition of oxidation in the presence of antioxidants. The degree of reduction in color is proportional to the capacity of antioxidant (Prior, Wu, & Schaich, 2005).

2.1.4.7. Metal ion Chelating Activity:

The ability of antioxidants to reduce Fe^{3+} can be assayed using ferrozine as an indicator (Dinis, Madeira, & Almeida, 1994). Ferrozine is a chromophoric chelator that strongly binds Fe^{2+} forming a stable complex with a high extinction coefficient at 562 nm (Dinis, Madeira, & Almeida, 1994). The Fe^{2+} concentration

is determined by using an extinction coefficient for the Fe [ferrozine]$_3^{2+}$complex (Decker & Welch, 1990). Measurement of reduction in color formation is directly related to the chelating activity of the chelator (Decker & Welch, 1990).

2.2. Enzyme Inhibitors:

Enzyme inhibitors are substances that decrease enzyme activity when present in an enzyme catalysed reaction medium.

2.2.1. Categories of Enzyme Inhibitors:

Enzyme inhibitors are classified as irreversible inhibitors and reversible inhibitors. The reversible type inhibitors are further divided into three groups competitive, non-competitive and uncompetitive.

2.2.1.1. Reversible Inhibitors:

1. ***Competitive Inhibitor:***

   Competitive inhibitor binds to the enzyme by competing with the substrate for the active site of the enzyme so binding of substrates by the enzyme for subsequent transformation to products is restrained. The HIV-1 protease inhibitors, LLEYSI and LLEYSL, were identified from thermolysin digests of oyster proteins (Harnedy & FitzGerald, 2012; T. G. Lee & Susumu, 1998). Both peptides were shown to act as competitive inhibitors of the HIV protease (Harnedy & FitzGerald, 2012).

2. ***Non-competitive Inhibitor:***

   Non-competitive inhibitors bind to sites other than the active sites of the enzyme and change the structural characteristic of the enzyme such that binding and transformation of substrates to products is impeded. Thus even if substrate is bound to enzyme, enzyme cannot catalyse. Angiotensin I-converting enzyme (Alemán, Giménez, Gómez Guillén, López de Lacey, López-Caballero, & Montero García, 2010) inhibitor, P4 peptide, [Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe], were isolated from chicken breast muscle extract (Saiga, Okumura, Makihara, Katsuda, Morimatsu, & Nishimura, 2006). P4 peptide is a non-competitive inhibitor

of ACE (Joseph Thomas Ryan, Reynolds Paul Ross, Declan Bolton, Gerald F. Fitzgerald, & Stanton, 2011).

3. ***Uncompetitive Inhibitor :***

   Uncompetitive inhibitor binds only to enzyme-substrate complex. This means the binding site of the inhibitor opens up after the substrate binds to the enzyme. Angiotensin I-converting enzyme inhibitor, IW, FY and AW were isolated from protease S digests of Wakame (*Undaria pinnatifida*) (Jao, Huang, & Hsu; Sato, Hosokawa, Yamaguchi, Nakano, Muramoto, Kahara, et al., 2002). These three peptides acted as an uncompetitive ACE inhibitor with 1.5, 42.3 and 18.8 as IC_{50} (μM) value (Jao, Huang, & Hsu; Sato, et al., 2002).

2.2.1.2. Irreversible Inhibitors:

Irreversible inhibitors react with enzymes and produce enzyme proteins that are not enzymatically active. Thus the enzyme loses its original state and cannot be regenerated. Poisons act by this mechanism. An irreversible serine protease inhibitor, termed baserpin, was purified for the first time from the skin secretions of toad *Bufo andrewsi* by successive ion-exchange and gel-filtration chromatography (Zhao, Jin, Wei, Lee, & Zhang, 2005). Baserpin is a potent inhibitor of bovine trypsin ($4.6\times106\ M^{-1}\ s^{-1}$), bovine chymotrypsin ($8.9\times106\ M^{-1}\ s^{-1}$) and porcine elastase ($6.8\times106\ M^{-1}\ s^{-1}$) (Zhao, Jin, Wei, Lee, & Zhang, 2005). The N-terminal sequence of baserpin is HTQYPDILIAKPXDK, which shows no similarity with other known serine protease inhibitors (Zhao, Jin, Wei, Lee, & Zhang, 2005).

2.2.2. Enzyme Inhibitors in Food and Human Health:

Certain diseases and deterioration of food are caused by the natural enzymes present in our food and living system. There are several studies to investigate and to produce natural enzyme inhibitors to curtail the adverse effects of endogenous enzymes (Soottawat Bejakul, Sappasiith Klomklao, & Simpson, 2009). Protease inhibitors, lipase inhibitors and Angiotensin I converting enzyme inhibitors are examples of enzyme inhibitors that have been studied extensively (Alemán, Giménez, Gómez Guillén, López de Lacey, López-Caballero, & Montero García, 2010).

2.2.2.1. ACE Inhibitor:

Angiotensin-converting enzyme [ACE, EC 3.4.15.1] is involved in the regulation of blood pressure. ACE acts via the formation of a vasopressor angiotensin II and bradykinin which acts as a vasodepresser. Recent research studies show that bioactive protein hydrolysates can play a vital role in lowering blood pressure by acting as an ACE inhibitor. The antihypertensive protein hydrolysates can be produced during gastrointestinal digestion, fermentation and maturation process, enzymatic hydrolysis and genetic recombination in bacteria (Hernández-Ledesma, Amigo, Ramos, & Recio, 2004). Protein hydrolysates with ACE inhibitor have been extracted from many sources like wheat, soy, milk, egg, corn, animal meat and marine products like seaweed, fish and shellfish (Arvind Kannan, Hettiarachchy, & Marshall, 2010; Joseph Thomas Ryan, Reynolds Paul Ross, Declan Bolton, Gerald F. Fitzgerald, & Stanton, 2011). There are some studies that shows consumption of food products or extracts with antihypertensive property significantly lowers the blood pressure to higher extents than is achieved with synthetic antihypertensive drugs (López-Fandiño, Recio, & Ramos, 2007).

2.2.2.2. Protease Inhibitor:

Proteases are enzymes that breakdown protein and peptide molecules. Thus due to the presence of some protease enzyme, essential proteins and peptide can be cleaved to non-essential or toxic molecules. Protease inhibitors are studied extensively as a replacement of antiviral agent for HIV disease, where virus encoded protease is targeted by the protease inhibitor (Irene T. Weber, Ying Zhang, & Tozser, 2009).

Protease inhibition in the food industry is desirable in food processing and preservation. Cheese making, brewing, bread making, meat tenderization, etc., involves proteolysis. When proteolytic enzyme activity is controlled during the process, best quality products can be obtained. Proteolysis during storage also increases the availability of nutrients for microbial growth (Aksnes, 1989). Proteolytic action in marine foods is a major factor that affects the shelf life of these food products (Aksnes, 1989).

Alpha amylase enzyme plays a vital role in bread making. Sticky dough is formed due to extensive degradation of starch by high level of alpha amylase. Thus to have a controlled

action of alpha amylase during breading making alpha amylase inhibitors can be used (Oort, 2009). The heat treatment process could affect the quality of bread. Use of alpha amylase inhibitor will be a good replacement for other old techniques. Alpha amylase inhibitor also has medicinal advantages; alpha amylase enzyme present in the digestive system can break down the essential starch molecules. This process is inhibited by alpha amylase inhibitor which helps in reducing the risk of obesity and diabetes mellitus. Ali, Houghton, and Soumyanath (2006) have studied α-amylase inhibitory activity of some Malaysian plant extracts used to treat diabetes, with particular reference to *Phyllanthus amarus*. There are many studies done on plants to extract alpha amylase inhibitor which act as an anti-diabetic agent (Etxeberria U, de la Garza AL, Campión J, Martínez JA, & FI, 2012).

2.2.2.3. Lipase Inhibitor:

Hyperlipidaemia directly or indirectly plays a role in causing diseases like obesity, diabetes, hypertension and cardiovascular problems. Obesity seems to be a major issue globally as it is linked to the cause of multiple diseases. The main reason for obesity is imbalance between energy intake and expenditure. Physical activities help in increasing expenditure. Researchers expect to develop inhibitors of nutrient digestion and absorption.

Orlistat, Sibutramine and Rimonabant are few commercially available lipase inhibitors which have been approved by FDA and Europe (Bray, 2011). Synthetic drugs such as rimonabant can cause many unpredictable side effects (Bray, 2011). Thus there are many researches going on to produce useful naturally derived lipase inhibitors from plant, animal and microbial sources (de la Garza, Milagro, Boque, Campión, & Martínez, 2011). Tables 2.1 and 2.2 lists few plant derived lipase inhibitors

Table: 2.1. Plant derived lipase inhibitors

Name of lipase inhibitor	Plant Source	Reference
Scabiosaponins	*Scabiosa tschiliensis*	Zheng and Koike (2004)
Sessiloside and chiisanoside	Leaves of *Accanthopanax sessiliflorus*	Yoshizumi (2006)
Cyclocariosides	Leaves of *Cyclocarea paliurus.*	Kurihara.H (2003)
Carnosic acid	Leaves of *Salvia officinalis*	Moreno (2006)
CT-II extract	Cassia nomame fruits	Hatano.T (1997)
Peanut shell extract	Arachis hypogaea or peanut shell	Ninomiya.K (2004)

Table: 2.2. Lipase inhibitors derived from Microbial source

Name of lipase inhibitor	Microbial Source	IC_{50} value	Reference
Lipstatin	*Streptomyces toxytricini*	0.14µM	Weibel (1987); Hochuli (1987)
Panclicins	*Streptomyces sp. NR 0619*	2.9, 2.6, 0.62, 0.66 and 0.8 µM	Mutoh, Nakada, Matsukuma, Ohshima, Yoshinari, Watanabe, et al. (1994)
Ebelactones	Actinomycetes strain G7-Gl, closely related to *Streptomyces aburaviensis*	3 and 0.8 ng/mL	Umezawa (1978)
Esterastin	*Streptomyces lavendulae* strain MD4-C1	0.2 ng/mL	Umezawa (1978)
Caulerpenyne	*Caulerpa taxifolia*	2 and 13 µM	Tomoda (2002)
Vibralactone	*Boreostereum virans*	0.4 µg/mL	Z. Liu, Dong, Xu, Zeng, Song, Zhao, et al. (2008)

Marine algae derived enzyme inhibitors like Caulerpenyne and Pholorotannins have been shown to be effective replacement to synthetic enzyme inhibitors in food (Faid, 2011). Pholorotannins also helps to reduce or prevent rancidity in fish oil (Faid, 2011).

2.3. Antimicrobial activity:

Microbial activity and metabolism can have important consequences activity and metabolism can have important consequences for food quality and safety as well as human health. Microbial metabolism can lead to food spoilage or release of toxins into foods, thus the need for the discovery and use of antimicrobials as food preservatives. The use of antimicrobial substance and preservatives is common practise in the food industry; nonetheless, the growing resistance of microorganisms to conventional antimicrobials, particularly antibiotics, is a foremost concern that has stimulated research to discover novel and more effective alternatives to curtail the adverse effects of microorganisms in foods. Thus the search for antimicrobial compounds from "natural" source (such as food materials and food products) has been increasing in recent years.

2.3.1. Antimicrobial protein hydrolysates:

There have been many studies on antimicrobial protein hydrolysates from several plant and animal sources (David Andreu & Rivas, 1998). Antimicrobial protein hydrolysates are produced by various methods. Recently enzymatic hydrolysis seems to be the most preferred method for production of antimicrobial protein hydrolysates. Antimicrobial protein hydrolysates usually comprise of bioactive peptides with up to 50 amino acids of which 50% are hydrophobic (Najafian & Babji, 2012). The antimicrobial peptides derived by enzymatic hydrolysis are usually below 10 kDa in size (Bulet, Stöcklin, & Menin, 2004; Z. Liu, et al., 2008). Examples of antimicrobial peptides derived from animal sources are listed in Table: 2.3

Table: 2.3. Antimicrobial Peptides Derived from Animal sources:

Antimicrobial peptide	Source	Antimicrobial activity	Reference
Casecidins and Isracidin	Casein protein	Gram-negative and Gram-positive bacteria	(Lahov & Regelson, 1996)
Lysozyme hydrolysates	Hen egg white Lysozyme	Gram-negative and Gram-positive bacteria	(Mine, Ma, & Lauriau, 2004)
Lactoferricin	Lactoferrin	*Bacillus, Escherichia coli, Klebsiella, Listeria, Proteus, Pseudomonas, Salmonella, Streptococcus,* and *Candida*	(Gifford, Hunter, & Vogel, 2005)

2.3.2. Marine Derived Antimicrobial Peptides:

Marine derived antimicrobial peptides exhibit a broad spectrum of antimicrobial activity. For example, the antimicrobial peptide from oyster, CgPep33 [Cys, Leu, Glu, Asp, Phe, Tyr, Ile, and Gly] inhibited growth of bacteria like *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, and fungi such as Botrytis cinerea, and Penicillium expansum* (Z. Liu, et al., 2008). CgPep33 have IC_{50} value ranging from 18.6 - 48.2 µg/mL against the above mentioned bacteria and fungi (Z. Liu, et al., 2008). T. G. Lee and Susumu (1998) investigated two antimicrobial peptides [Leu-Leu-Glu-Tyr-Ser-Ile and Leu-Leu-Glu-Tyr-Ser-Leu] from oyster and found them to have antiviral activity such as inhibition of HIV-1 protease. The antimicrobial peptide isolated from American lobster, Gln-Tyr-Gly-Asn-Leu-Leu-Ser-Leu-Leu-Asn-Gly-Tyr-Arg, exhibited bacteriostatic activity against some gram-negative bacteria [*Micrococcus luteus, Aerococcu viridans, Vibrio* sp, *Vibrio parahaemolyticus, Halomonas* sp and *Enterobacter aerogenes*] and both protozoastatic and protozoacidal activity against two scuticociliate parasites [*Mesanophrys* sp and *Anophryoides haemophila*] (Battison, Summerfield, & Patrzykat, 2008).

Antimicrobial peptides like arasin 1 and callinectin from crab species like the spider crab and blue crab inhibited the growth of *Corenebacterium glutamicum* and Gram-negative

bacteria [*E.coli* D31] (Khoo, Robinette, & Noga, 1999; Stensvag, Haug, Sperstad, Rekdal, Indrevoll, & Styrvold, 2008). Several α-helical antimicrobial peptides have been isolated from fish. Table: 2.4 list few antimicrobial peptides derived from fish.

Table: 2.4. Fish derived Antimicrobial Peptides

Source	Antimicrobial peptide*	Micro-organism inhibited	Reference
Hagfish	Myxinidin [3.5kDa,12aa]	*Salmonella enterica, Escherichia coli, Aeromonas salmonicida, Yersinia ruckeri* , and *Listonella anguillarum*	Subramanian, Ross, and MacKinnon (2009)
Red Sea Moses sole	Pardaxins [3.3kDa, 33aa]	Gram positive and gram negative bacteria	Oren and Shai (1996)
Winter flounder	Pleurocidins [2.7kDa, 25aa]	Gram positive and gram negative bacteria	Cole, Weis, and Diamond (1997)
Mudfish	Misgurin [2.5kDa,21 aa]	*Bacillus subtilis, Staphylococcus aureus, Streptococcus mutans, Streptococcus pneumoniae, Escherichia coli, Serratia sp., Pseudomonas putida* and *Salmonella typhimurium. Candida albicans, Saccharomyces cerevisiae* and *Cryptococcus neoformance*	C. B. Park, Lee, Park, Kim, and Kim (1997)
Catfish	Parasin [2.0kDa,19 aa]	*Bacillus subtilis, Staphylococcus aureus, Streptococcus mutans, Pseudomonas putida, Escherichia coli, Salmonella typhimurium, Serratia sp. Cryptococcus neoformans, Saccharomyces cerevisiae, Candida albicans*	I. Y. Park, Park, Kim, and Kim (1998)
Rainbow trout	Oncorhyncin II [69 aa]	Gram positive and gram negative bacteria	Smith, Fernandes, Jones, Kemp, and Tatner (2000)

Red sea bream	Chrysophsins [2.95kDa,20-25 aa]	*Escherichia coli, Bacillus subtilis*, and fish and crustacean pathogens	Iijima, Tanimoto, Emoto, Morita, Uematsu, Murakami, et al. (2003)
Atlantic halibut	Hipposin [5.5kDa, 51aa]	*Escherichia coli, Vibrio anguillarum* and *Bacillus subtilis*	Birkemo, Lüders, Andersen, Nes, and Nissen-Meyer (2003)

*[Approximate molecular weight of peptide and number of amino acids {aa}].

The ability of fish to survive in a pathogenic environment is mainly due to the existence of antimicrobial peptides in the epidermal layer of fish. In recent years there have been numerous studies conducted on biological active substances from fish skin with antimicrobial activity. A recent review by Rajanbabu and Chen (2011) shows that almost all fish derived antimicrobial peptides directly exhibit antibacterial activity.

2.3.3. Microbial Growth enhancers:

Although production of antimicrobial from food sources is important for the food industry; it is also essential to develop approaches based on natural and innocuous products that can maintain or enhance the survival of useful microbes like probiotic bacteria and fermentative microorganisms. In food science it is also essential to investigate protein hydrolysates for their efficacy in increasing the survival of probiotic bacteria in food and also in increasing the survival of microorganism used for fermentation specifically during their microbial fermentation process to enhance the development of probiotic food. Thus, production of protein hydrolysates from natural compounds for use as nitrogen source for microbial growth media is also an area of active research (Vasileva-Tonkova, Nustorova, & Gushterova, 2007). Peptones are one of the most important nutrient sources in bacterial medium. Vasileva-Tonkova, Nustorova, and

Gushterova (2007) studied the use of peptones from protein hydrolysis of collagen waste for bacterial growth. Protein hydrolysates produced from tropical tuna with alcalase performed effectively as cellular growth factors on fibroblastic cell and as nitrogen source in microbial growth media (Guerard, Ravakkec-Ple, Broise, Binet, & Dufosse, 2002).

Probiotic bacteria serve as a beneficial microorganism in food. Survival of probiotic bacteria is slow due to their low level of proteolytic activity and also traditional culture suppresses the growth of probiotic bacteria (McComas & Gilliland, 2003). Minimum 10^7 viable cells/g ml of probiotic bacterial cells has to be maintained until the consumption of food. Commonly known nitrogen source like yeast extract, liver extract, peptones and corn steep liquor cause undesirable flavour (Anderson & Elliker, 1953). Study by (McComas & Gilliland, 2003) shows the use of whey protein hydrolysate in yogurt significantly increased the growth of *Bifidobacterium longum* S9, *L.acidophilus* O16, and *L.acidophilus* L-1.

2.3.4. Methods used to test antimicrobial activity:

There are various methods available for testing antimicrobial activity. Example are the agar diffusion method, the agar and broth dilution method, gradient plates and spiral plating methods, turbidimetric assays, and inhibition curves method (Davidson, Enrique, Mickey, & Aurelio Lopez-Malo, 2005).

2.3.4.1. Zone Inhibition Assay:

The agar diffusion method which is also known as inhibition zone assay is generally used to study the antimicrobial activity of peptides. The antimicrobial peptides are placed on sterilised filter paper or in wells made in agar plate which is already inoculated with microorganism (Davidson, Enrique, Mickey, & Aurelio Lopez-Malo, 2005).

“Muller Hinton Agar” is a transparent medium well suited and most widely used for studying anti-microbial activity. On an agar plate made with this medium, the organism against which the test is being done is spread and the test compound or extract is placed as a disc at the centre. If the test sample has anti-microbial activity, it manifests as a clear zone (i.e., growth inhibition) around the disc. It has been shown that certain biologically

active compounds exhibit anti-microbial activity against bacteria species like *Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Lactobacillus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella paratyphi, Salmonella typhi, Staphylococcus aureus* and *Vibrio cholera* (Kuppulakshmi, Prakash, & Gunasekaran, 2008).

The diameter of inhibition zone is measured to estimate the inhibition activity of the antimicrobial peptide. To make an accurate study, the minimum inhibition concentration (MIC) value is determined. Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001). The determination of the MIC is possible in agar diffusion method, but it is easier in other methods (Davidson, Enrique, Mickey, & Aurelio Lopez-Malo, 2005).

2.3.4.2. Inhibition Curve:

The inhibition curve method is also known as the "time kill curves" method. According to NCCLS (2002), the method uses a non-selective broth medium such as trypticase soy broth to which single concentration of antimicrobial agent is added. The concentration of antimicrobial is determined from methods like agar diffusion, agar and broth dilution, gradient plates, spiral plating (methods which are collectively termed as "endpoint methods"). The microorganism which is used as the test organism to verify antimicrobial activity is diluted such that their final concentration is approximately log 5.7 CFU/mL in the test medium (NCCLS, 2002). The inhibition curve technique demonstrates lethality. One drawback of this techniques is that no single statistic is produced to compare such treatments as MIC; It is also labour intensive and expensive (Michael Davidson, Sofos, & Branen, 2005).

2.3.4.3. Turbidimetric Assay:

Turbidity assay method determines antimicrobial effectiveness over time and it measures turbidity increases spectrophotometrically. The spectrophotometric detection range generally requires log 6.0 to 7.0 CFU/mL (Brock, Smith, & Madigan, 1984). Microbial growth below log 5 CFU/mL is undetectable using spectrophotometer (Davidson,

Enrique, Mickey, & Aurelio Lopez-Malo, 2005). A method proposed by Marwan and Nagel (1986) is based on the time for a microbial population to reach a specific turbidity in the presence of an antimicrobial agent divided by the time for the same population to reach the turbidity in the absence of the antimicrobial agent. This parameter is referred as Relative Effectiveness (RE) value (Davidson, Enrique, Mickey, & Aurelio Lopez-Malo, 2005) . A linear graph is obtained when 1/RE was plotted against concentration of inhibitor. Lambert and Pearson (2000) determined MIC using optical density and the method involved comparing the area under the graph obtained by plotting the optical density versus time with various concentrations of the test samples or without. The wavelength usually used in these methods range between 400 to 660 nm.

Chapter 3: MATERIALS AND METHODS

3.1. Materials Required:

3.1.1. Biological Specimen:

Dogfish skin was purchased from OCN Import fish market, Montreal. The skins were stored at -20˚C until used for investigation.

3.1.2. Microbial cultures:

DH5α *E.coli*, *Bacillus subtilis* and *Lactococcus lactis* strains were obtained from the collection of Don Niven, McGill University, QC, Canada.

3.1.3. Enzyme:

Bovine Pancreas α-Chymotrypsin and Porcine Pancreas Trypsin are from ICN Biomedicals, Inc. Type 2 Porcine Pancreatic Lipase are purchased from Sigma, Steinheim, Germany. Papain purchased from ACROS Organic, New Jersey, USA.

3.1.4. Reagents:

HPLC grade Acetone, Hexane, Acetonitrile, Ethanol from Fisher Scientific, New Jersey, USA. EDTA, Trichloroacetic acid (TCA), Thiobarbutric acid (TBA) is purchased from Fisher Scientific, New Jersey, USA. 1,1-diphenyl-2-picrylhydrazyl [DPPH], 2-deoxyribose, 4-nitrophenylpalmitate (PNP), and Glutathione (GSH) are purchased from Sigma, Steinheim, Germany. BCA assay kit is purchased from Thermo Scientific, Rockford, USA. Ferrous Sulphate ($FeSO_4$) is purchased from Anachemia Science, Montreal, Canada. Pyrogallol, Potassium ferricyanide, 3-(2-pyridyl)-5 and 6-bis (4-phenyl-sulfonic acid)-1, 2, 4-triazine (Ferrozine) are purchased from ACROS Organic, New Jersey, USA. Nutrient broth, Trypticase soy broth and Agar were obtained from Difco Co, Becton Dickson.

3.2. Preparation of Sample:

The dogfish skin was cleaned with distilled water, towelled dry with paper towels, and chopped into very small pieces. Then it was comminuted into powder in a warring blender with liquid nitrogen. The powdered skin was subsequently defatted at -20°C using HPLC grade acetone and hexane at different ratios (i.e., 3:2, 1:3, 1:1 and 3:1) sequentially at 1 hr intervals. The defatted skin was air dried at ambient temperature in a fume hood overnight.

3.2.1. Enzyme Hydrolysis:

The dried defatted dogfish skin powder was hydrolysed using three different enzymes, namely α-chymotrypsin, trypsin and papain. Different sample buffers were used for different enzyme hydrolysis, based on the pH optima for the particular enzymes. The sample buffers used are 50 mM Tris HCl (containing 20 mM $CaCl_2$, pH 7.8) for α-chymotrypsin hydrolysis; 50 mM Tris HCl (containing 20 mM $CaCl_2$, pH 8) for trypsin hydrolysis; and 50 mM Tris HCl pH 7 for papain hydrolysis. To carry out controlled process, selection of enzyme inactivation method and optimisation of enzyme hydrolysis was performed as follows:

3.2.1.1. Inactivation of Enzyme after Hydrolysis:

Inactivation of enzyme after hydrolysis is necessary to prevent excessive and uncontrolled hydrolysis. This is done by either heat or acid inactivation, or by filtration to remove the enzyme from the reaction mixture. A homogenate of the dried defatted dogfish skin powder was prepared in the sample buffer (1:5) and hydrolysed for 4 h using 1% (w/w) of the enzyme. After the enzyme hydrolysis, the hydrolysates are treated with different methods like heat inactivation method, inactivation by acid and removal by filtration method. The treatment which produced no or the lowest residual enzyme activity was selected for stopping the enzyme hydrolysis.

a) Inactivation by Heat Treatment:

Enzyme inactivation was done by heating the protein hydrolysates at 90°C for 15 min.

b) Inactivation by acid treatment:

Enzyme inactivation was done by using TCA to a final concentration of 5% TCA

c) Filtration:

Filtration was performed using 10 kDa Amicon Ultra Centrifugal Filter tubes (Millipore, Ireland) with centrifugation at 5,100 g for 20 min at 4°C.

After the inactivation or removal the enzyme, the hydrolysates were freeze dried and stored at -18^{o}C, and used for studies to verify various biological properties as described in the sections below.

3.2.1.2. Optimisation of Enzyme Hydrolysis:

The dried defatted dogfish skin powder was stirred thoroughly into a homogenate in the sample buffer in a ratio of 1:5 (w/v). The extraction of protein hydrolysates was optimized by varying the time of hydrolysis (2 and 4 h) and the enzyme concentration (i.e., 0.05%, 0.1%, 0.2%, 0.5% and 1%, based on the weight of the substrate), at 37°C temperature condition. DPPH assay (described under 3.3.1.1) was performed for the different protein hydrolysates to determine the best conditions for hydrolysis to obtain protein hydrolysates with the most DPPH radical inhibition activity. This optimisation of hydrolysis condition was performed by using only α-chymotrypsin enzyme. The reaction mixture was centrifuged with a speed of 6,800 g for 10 min at 4°C with a Beckman J2-21 Centrifuge.

3.2.2. Protein Estimation:

Protein concentration was estimated using BCA assay kit with 2 mg/mL of BSA as standard. The assay was performed with 0.1 ml of different known concentration of standard protein, to which 2mL of BCA reagent was added and incubated at 37°C for 30mins, then read at 562nm. A linear plot was obtained, from which the unknown protein concentration of the sample was estimated.

3.3. Methods to Determine the Biologically Active Peptide:

The sample extract was verified for antioxidant, antimicrobial and enzyme inhibitory activities as follows:

3.3.1. Measuring Antioxidant Activity of the Protein Hydrolysates:

3.3.1.1. DPPH Free Radical Scavenging Activity:

The DPPH free radical scavenging activity of the fish protein hydrolysates was studied using method of H. Liu, Wang, and Wang (2011) with slight modifications. The sample buffer was used to dilute the sample extract. Next, 1 mL of 1 mM DPPH in 80% ethanol was added to 1 mL of different concentrations of the sample extract. This reaction mixture was vortexed for 1 min to ensure thorough mixing and then incubated in dark for 30 min at room temperature (≈25°C). The absorbance was measured at 517 nm in a Beckman Coulter DU800 UV/VIS spectrophotometer. DPPH radical scavenging activity was calculated as:

$$\textit{DPPH Radical scavenging capacity (\%)} = [1-\{(A_i - A_j)/ (A_c - A_d)\}] \times 100\ \%$$

where, A_i is the absorbance of 1 mL extract mixed with 1 mL of DPPH solution, A_j is the absorbance of 1 mL extract mixed with 1mL DPPH free radical solvent, A_c the absorbance of 1mL DPPH mixed with 1 mL of the sample buffer, and A_d the absorbance of 1 mL of the sample buffer and 1 mL of 80% ethanol. The sample extract concentration required to scavenge 50% DPPH radials (IC_{50} value) was calculated from a plot of scavenging activity against the extract concentration. Glutathione was used as positive control.

3.3.1.2 Hydroxyl Radical Scavenging Antioxidant Activity:

The method of Yang et al., (Yang, Ke, Hong, Zeng, and Cao (2011) was used with slight modifications to study the hydroxyl radical scavenging capacity of the samples. The reaction mixture consisted of 0.2 mL of 10 mM $FeSO_4$–EDTA, 0.5 mL of 10 mM 2-deoxyribose, 0.9 mL of sodium phosphate buffer (pH 7.4), and 0.2 mL of the sample were thoroughly mixed in a test tube. Hydrogen peroxide (0.2 mL, 10 mM) was then added and the reaction mixture was incubated at 37°C for 1 h. After incubation, 1.0 mL of 2.8% TCA (w/v) and 1.0 mL of 1.0% 2-thiobarbituric acid (TBA) were added to the reaction mixture in the test tubes and boiled for 15 min. After cooling the mixture, the absorbance was measured at 532 nm in a Beckman Coulter DU800 UV/VIS spectrophotometer.

Ascorbic acid was used as positive control. The results were calculated as the percentage of free radical scavenging effect according to the following formula:

$$\textit{Hydroxyl radical scavenging effect (\%)} = [1- \{(S - S_B)/ (C - C_B)\}] \times 100 \%$$

where, S is the absorbance of the sample, S_B is the blank sample, C is the control and C_B the blank control, respectively.

3.3.1.3. Superoxide Anion Scavenging Assay:

The assay was carried out using a modified version of the method by Marklund and Marklund (1974), which is based on the inhibition of pyrogallol auto-oxidation. The assay mixture comprised of 2.6 mL Tris HCl buffer (50 mM, pH 8) and 0.3 mL of the sample extract. The reaction mixture was preincubated at 25°C for 20 min, after which the reaction was initiated by the addition of freshly prepared 0.1 mL pyrogallol (3 mM pyrogallol in 10 mM HCl). Auto- oxidation of pyrogallol was measured spectrophotometrically at 325 nm. The absorbance of each extract was recorded at 1min interval for 10 min. GSH was used as positive control. The scavenging rate was calculated according to the following equation:

$$\textit{Superoxide radical-scavenging rate (\%)} = [1- \{(A_1 - A_2)/ (A_0 - A_b)\}] \times 100$$

where A_b is the absorbance of control blank (2.6 mL of 50 mM Tris HCl buffer, pH 8) + 0.3 mL of sample buffer + 0.1 mL of 10 mM HCl), A_0 is the absorbance of the control (without sample) (2.6 mL of 50mM Tris HCl buffer, pH 8) + 0.3 mL of sample buffer + 0.1 mL of pyrogallol solution), A_1 is the absorbance in the presence of the sample (2.6 mL 50mM Tris HCl buffer, pH 8) + 0.3 mL of sample extract+ 0.1 mL of pyrogallol solution), and A_2 was the absorbance of the sample without pyrogallol (2.6 mL of 50mM Tris HCl buffer, pH 8) + 0.3 mL of sample buffer + 0.1 mL 10 mM HCl).

3.3.1.4. Reducing Power Assay:

The method described by Singh and Rajini (2004) was used to determine the reducing power of the extracts. Total 1 mL aliquot of the extract of different concentration was added to 2.5 mL of 0.2 M phosphate buffer pH 6.6, and 2.5 mL of 1% (w/v) potassium

ferricyanide were added and incubated at 50°C for 30 min. Further, 10% TCA (w/v) added to terminate the reaction. This mixture was centrifuged at 3,000 g for 10 min. About 2.0 mL of the supernatant was mixed with 2.0 mL distilled water and added to 0.5 ml of ferric chloride (0.1% w/v) solution and the absorbance was measured at 700 nm. GGH was used as positive control. The increase in absorbance of the sample compared to control was used as a measure of the reducing power of the sample.

3.3.1.5. Metal ion Chelating activity:

The method of Decker and Welch (1990) was used with slight modification for measuring metal ion chelating activity of the sample. About 1 mL of various concentrations of the sample extract was first mixed with 3.7 mL of distilled water. Then it was reacted with a solution containing 0.1 mL of 0.2 mM $FeCl_2.4H_2O$ and incubated for half a minute. This reaction was then mixed with 0.2 ml of 5 mM 3-(2-pyridyl)-5, 6-bis (4-phenyl-sulfonic acid)-1, 2, 4-triazine (Ferrozine). After 10 min of incubation at room temperature (≈ 25^oC), the optical density of the Fe^{2+}- Ferrozine reaction complex was measured spectrophotometrically at 562 nm. EDTA was used as positive control. Metal ion chelating activity was calculated as follows:

$$\textit{Chelating activity was calculated (\%)} = [1-\{(M_i - M_j)/ (M_c - M_d)\}] \times 100\ \%$$

where M_i – is the absorbance of sample (1 mL of sample extract+ 3.7 m of distilled water + 0.1 mL of $FeCl_2.4H_2O$ solution+ 0.2 mL of Ferrozine solution), M_j - is the absorbance of sample blank (1 mL of sample extract+ 3.8 mL distilled water + 0.2mL of Ferrozine solution), M_c - is the absorbance of control (0.1 mL of $FeCl_2.4H_2O$ solution+ 4.7 mL of distilled water + 0.2 mL of Ferrozine solution), M_d - is the absorbance of control blank (0.1 mL of $FeCl_2.4H_2O$ solution+ 4.9 mL of distilled water).

3.3.2 Enzyme inhibitor activity:

3.3.2.1. Lipase enzyme Inhibitor:

Lipase inhibitor activity was studied using the method of Slanc, Doljak, Kreft, Lunder, Janeš, and Štrukelj (2009) and slight modification. An enzyme solution (5 mg/mL) was

prepared from type 2 porcine pancreatic lipase. A 10 mM solution of 4-nitrophenylpalmitate (PNP), was prepared in HPLC Grade acetonitrile and diluted to 3.33mM using Ethanol (Slanc, Doljak, Kreft, Lunder, Janeš, & Štrukelj, 2009). The composition of the reaction mixture was: 100 µL of 3.3 mM PNP, 1580 µL of 75 mM Tris-HCl buffer (pH = 8.5), 200 µL of extract (different concentration was used) and 120µL of enzyme solution. The mixture was incubated at 37 °C for 25 min before the substrate was added. In the positive control, the extract was replaced with the same volume of sample buffer. The absorbance was measured spectrophotometrically at 405 nm.

Lipase enzyme inhibitory activity (%) was calculated as follows:

$$Inhibition\ (\%) = [1 - \{(A_5 - A_6)/(A_7 - A_8)\}] \times 100$$

where, A_5 is the absorbance of incubated solution containing the sample, substrate and lipase, A_6 is the absorbance of incubated solution containing sample and substrate, A_7 is the absorbance of incubated solution containing the substrate and lipase, and A_8 is the absorbance of incubated solution containing the substrate only.

3.3.2.2. Alpha Chymotrypsin inhibitor assay:

The inhibition of α-chymotrypsin was studied according to the method specified in Sigma Aldrich protocol. An enzyme solution of 2 mg/mL of α-chymotrypsin was prepared in 1mM HCl. The substrate was 1.18 mM BTEE and 1 mg/mL of soybean trypsin inhibitor prepared in the appropriate sample buffer as described previously.

Different volumes of the inhibitor and sample extracts were transferred into test tubes and to this added 500 µL of the enzyme solution. The reaction volume was made up to 10 mL using 80 mM Tris buffer (containing 100 mM $CaCl_2$, pH 7.8) and incubated at 25°C for 5 min to constitute the reagent A.

Next, the following reagents, 80 mM Tris buffer (containing 100 mM $CaCl_2$, pH 7.8) and 1.4 mL of 1.18 mM BTEE were pipetted into suitable cuvets. The reaction mixture was mixed thoroughly by end-over-end inversion to constitute reagent B. Next, 0.1 mL of reagent A was added to reagent B and immediately mixed by inversion, and the

absorbance at 256 nm was recorded at 15 sec intervals for 5 min. Control for the assay was prepared using sample buffer. Blank solution for control and test was prepared using 1mM HCl.

α-chymotrypsin inhibitory activity (%) was calculated as follows:

$$Inhibition\ (\%) = [1 - \{(A_i - A_j)/(A_k - A_l)\}] \times 100$$

where, A_i is the absorbance of the incubated solution containing sample, substrate and α-chymotrypsin; A_j is the absorbance of incubated solution containing the sample and the substrate; A_k is the absorbance of the incubated solution containing the substrate and α-chymotrypsin, and A_l is the absorbance of the incubated solution containing the substrate.

3.3.3. Antimicrobial activity:

Antimicrobial activity of the protein hydrolysate was verified against three microorganisms, DH5α-*E.coli* strain, *Bacillus subtilis* and *Lactococcus lactis.*

3.3.3.1. Revival of Culture:

DH5α *E.coli* and *Bacillus subtilis* were revived using nutrient broth; while trypticase soy broth was used to revive *Lactococcus lactis.* The microorganisms were allowed to multiply in their respective media by incubating them at 37^{o}C for 24 h.

Table: 3. Microorganism used for antimicrobial test

No	Organism	Solid/ Liquid Medium for growth
1	DH5α- *E.coli*	Nutrient Agar/Broth
2	*Bacillus subtilis*	Nutrient Agar/Broth
3	*Lactococcus lactis*	Trypticase Soy Agar/Broth

3.3.3.2. Disc Diffusion Method:

Initially, antimicrobial activity of the protein hydrolysate was assayed qualitatively against all three microorganisms in their respective agar medium (nutrient agar for DH5α

E.coli, *Bacillus subtilis* and trypticase soy agar for *Lactococcus lactis*). Cultures grown overnight were serially diluted (dilution factor 10^1) and spread on their respective agar plates. Paper discs of 6 mm were placed at the centre of each plate. To the discs, 10µL of the protein hydrolysate was added. The plates were incubated at 37^{o}C for 24 h and observed for the zones of inhibition. Buffers used for the protein hydrolysates were used as negative control to verify their antimicrobial activity, if any. Polymyxin B was used as a positive control.

3.3.3.3. Survival Assay:

In this method, the cells were directly treated with the protein hydrolysates. 400µL of an overnight culture (DH5α-*E.coli* strain, *Bacillus subtilis* and *Lactococcus lactis*) was centrifuged at 5,000g for 5 min at 25˚C to form the pellet. Pellets were redissolved in 500µL protein hydrolysate and vortexed. Samples were taken at 0, 4, 48, and 96 h interval for the survival analysis.

Survival analysis was done by the following steps:

a) Aliquots of sample were diluted for 10 folds using specific diluents (Nutrient broth and Trypticase soy both) for their respective cultures (DH5α-*E.coli*, *Bacillus subtilis* and *Lactococcus lactis*). Plating was done on Petri film dish using the drop plating method.
b) After overnight incubation at 37^{o}C enumeration of the viable cells were done.

3.4. Statistical Analysis:

All experiments were performed in triplicate. Statistical analyses were performed using JMP statistical program (JMP 8.0, SAS Inc., Cary, NC, United States). Paired t-test was used to evaluate the significant differences or similarity among the hydrolysates at $p < 0.05$.

Chapter 4: RESULT AND DISCUSSION

4.1. Optimisation of protein hydrolysis conditions:

The optimisation of reaction conditions for the extraction of protein hydrolysates (peptides) was studied based on the hydrolysates obtained by α-chymotrypsin treated sample. The Figure 4.1.a demonstrates which treatment attained the most enzyme inactivation.

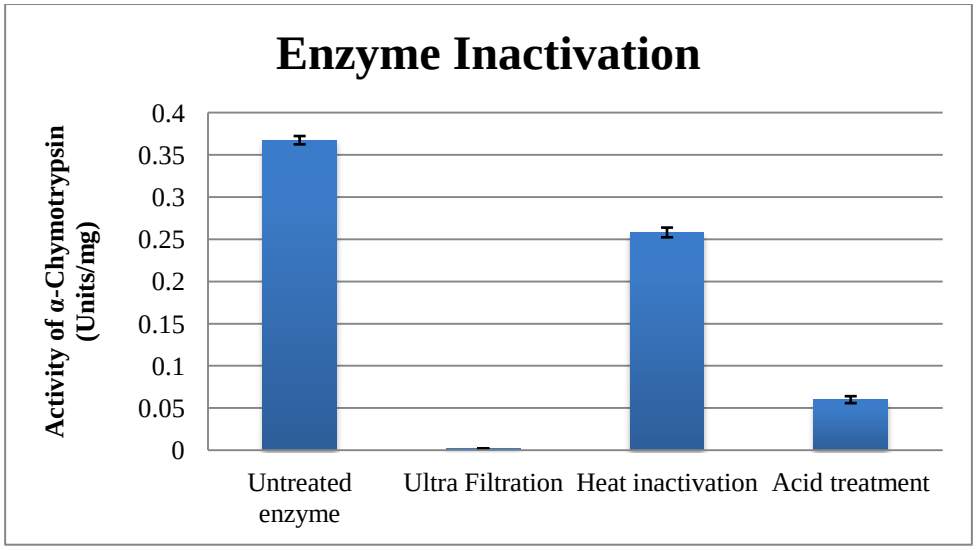

Figure: 4.1.a. Investigation of different treatments for enzyme inactivation

From the above figure it was inferred that protein hydrolysate after treating with ultra-centrifugal filtration had practically no residual enzyme activity suggesting that all or most of the enzymes was removed by filtration. Furthermore, that the filtration method would not cause any physical damage to protein hydrolysates compared to heat and acid treatment. Thus the filtration method was selected for enzyme removal after enzyme hydrolysis.

Optimization of the preparation of the protein hydrolysates was done by varying time and enzyme concentration, while keeping temperature and pH constant at 37˚C and at pH 7.8. The hydrolysates were verified for DPPH radical scavenging activity.

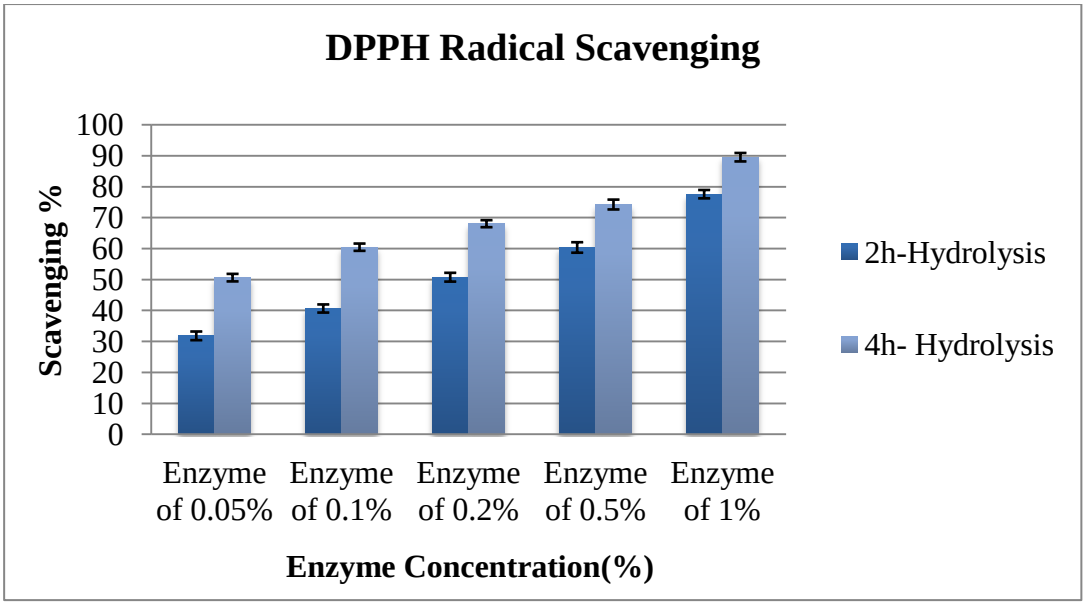

Figure: 4.1.b. DPPH Radical Scavenging assay to estimate the hydrolysis conditions which produce the hydrolysates having highest scavenging activity.

DPPH radical scavenging assay is the most sensitive antioxidant assay compared to other antioxidant assay as it responds to even low concentrations of antioxidants (Marinova & Batchvarov, 2011). Thus the protein hydrolysates obtained by different enzyme concentration and different time period was assayed using DPPH Radical Scavenging test. From Figure 4.1.b it is evident that hydrolysis of dried defatted dogfish skin powder with 1% enzyme for 4h produced protein hydrolysates having highest DPPH scavenging activity. The conditions producing the protein hydrolysates with the most DDPH activity using α-chymotrypsin were selected for the hydrolysis with the other enzymes, i.e. papain and trypsin. The three different protein hydrolysates obtained by 1% of α-chymotrypsin, papain and trypsin hydrolysis for 4h at 37°C were further investigated for selected biological activities.

The optimization of enzyme hydrolysis was checked for statistical significance using Factorial method. The model presenting the response (DPPH scavenging activity) in terms of the factors (enzyme concentration and time) had high R^2 value (0.937) and was significant at $p < 0.05$. The model can be presented as

DPPH Scavenging activity (%) =46.069+7.509 ×Time+39.461×Enzyme concentration

Both factors, time and enzyme concentration were highly significant (with p value 0.0042 and 0.0002 respectively). However, change in enzyme concentration influence more on the DPPH radical scavenging property of the protein hydrolysate than the time of hydrolysis.

4.2. Protein determination by BCA method:

Total protein was determined by the bicinchoninic acid method. The purple coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. Bovine serum albumin (BSA) was used as a reference protein to obtain a standard curve that was used to determine the protein concentrations of the hydrolysates. The BCA assay was carried out in duplicates for all the samples.

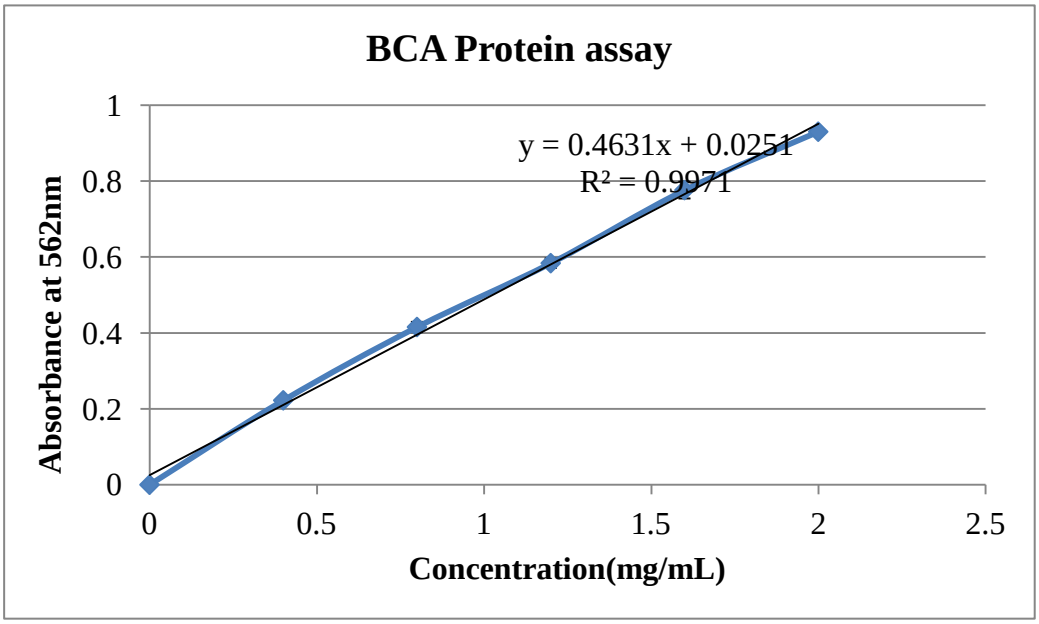

Figure: 4.2. BCA protein assay standard curve

Using the linear graph equation y= 0.4631x+ 0.0251 with R^2= 0.9971, the protein concentration of the protein hydrolysates were determined.

Table: 4.1. Protein concentration of the protein hydrolysates

Protein hydrolysates	Protein concentration (mg/mL)
α-Chymotrypsin treated protein hydrolysate (CPH)	0.131936
Trypsin treated protein hydrolysate (TPH)	0.17469
Papain treated protein hydrolysate (PPH)	0.13863

4.3. Antioxidant activity:

The extracted protein hydrolysates were verified for antioxidant activity using the different assays described below.

4.3.1. Reducing Power Activity:

Reducing power is an estimation of the capacity of a given compound to reduce ferric ion to ferrous ion (Yang, Ke, Hong, Zeng, & Cao, 2011) as an index of its antioxidant potential. In this assay ferric ion forms complex with ferricynaide, when an antioxidant is applied the ferric ion is reduced to its ferrous form, and the yellow color test solution changes to various shades of green and blue depending on reducing capacity of each compound (Gülçin, Büyükokuroğlu, Oktay, & Küfrevioğlu, 2003). The reducing ability of the antioxidant for the ferric ions is determined by measuring ferrous ion concentration, via the formation of Perl's Prussian blue color spectrophotometrically at 700 nm. The higher the absorbance at 700 nm, the greater the reducing power. It has been reported that species with reducing power have the ability to donate electrons to free radicals in order to form stable substances, thereby they can reduce oxidized intermediates of lipid peroxidation process (Yen & Chen, 1995).

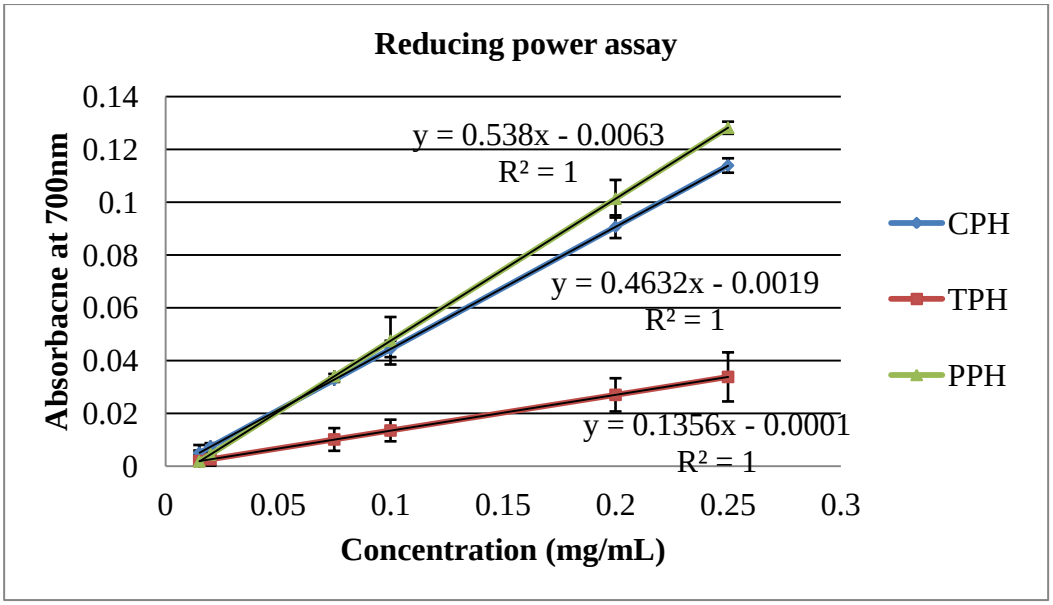

Figure: 4.3. Reducing power of different concentrations of CPH, TPH and PPH. Values represent the mean ± SD of triplicates.

Each peptide showed a linear relationship between activity and concentration: (TPH: y = 0.135x - 0.0001; CPH: y = 0.4632x - 0.0019; PPH: y = 0.538x - 0.006; P> 0.05). As the concentration of protein hydrolysates was increased, the ability of the protein hydrolysates to reduce ferric ion also increased (Figure: 4.3). Tuna fish protein hydrolysates studied by Yang, Ke, Hong, Zeng, and Cao (2011) also showed a concentration-dependent linear relationship with reducing power. This result is also consistent with the findings by Yen and Chen (1995), who reported a peanut hull extract having a linear relationship between reducing power and concentration (Yang, Ke, Hong, Zeng, & Cao, 2011).

In this study PPH showed the highest reducing power of 0.065 at 0.132 mg/mL, compared to 0.059 for CPH and 0.018 for TPH. The standard GSH shows reducing power of 0.519 at 0.1 mg/mL was much higher than the protein hydrolysates. It is suggested that increasing the concentration of the protein hydrolysates appropriately could increase the extent of reducing power to match that of GSH. While PPH showed the highest reducing power (as described above), TPH exhibited the least reducing power. From that statistical analysis using paired t-test the activity profile of PPH in reducing power capacity was significantly similar to CPH (P<0.2469), whereas TPH was significantly different from

the activity profile CPH ($P<0.0447$). The statistical comparison of activity profile of PPH vs TPH resulted $P<0.0607$.

4.3.2. Hydroxyl radical scavenging assay:

Hydroxyl radical is formed by a Fe^{3+}-EDTA-H_2O_2 complex according to Haber-Weiss reaction (Bektaşoğlu, Esin Çelik, Özyürek, Güçlü, & Apak, 2006). Due to EDTA chelating of Fe^{3+} the iron ion gets reduced to Fe^{2+}, which then reacts with H_2O_2 to form hydroxyl radicals. These radicals then react with α-deoxyribose, which degrades into chromophoric products that absorb light maximally at 532 nm. The addition of an antioxidant scavenges the hydroxyl radical, inhibiting the degradation of deoxyribose and subsequent color formation (Bektaşoğlu, Esin Çelik, Özyürek, Güçlü, & Apak, 2006). Hydroxyl radical is the most damaging oxygen radical, especially to proteins, DNA and lipid system (Yang, Ke, Hong, Zeng, & Cao, 2011). Scavenging and inhibiting this radical is of great importance in meats and plant systems to keep cell membranes intact. This radical can cause the crisp, fresh texture of vegetables to disintegrate.

This radical is also key in the oxidation of lipids, which is of great concern to the food industry. Not only this radical harmful to foods but also to human health, for it can cause diseases such as various cancers thus, it is beneficial to either prevent their formation, or to remove them (Yang, Ke, Hong, Zeng, & Cao, 2011).

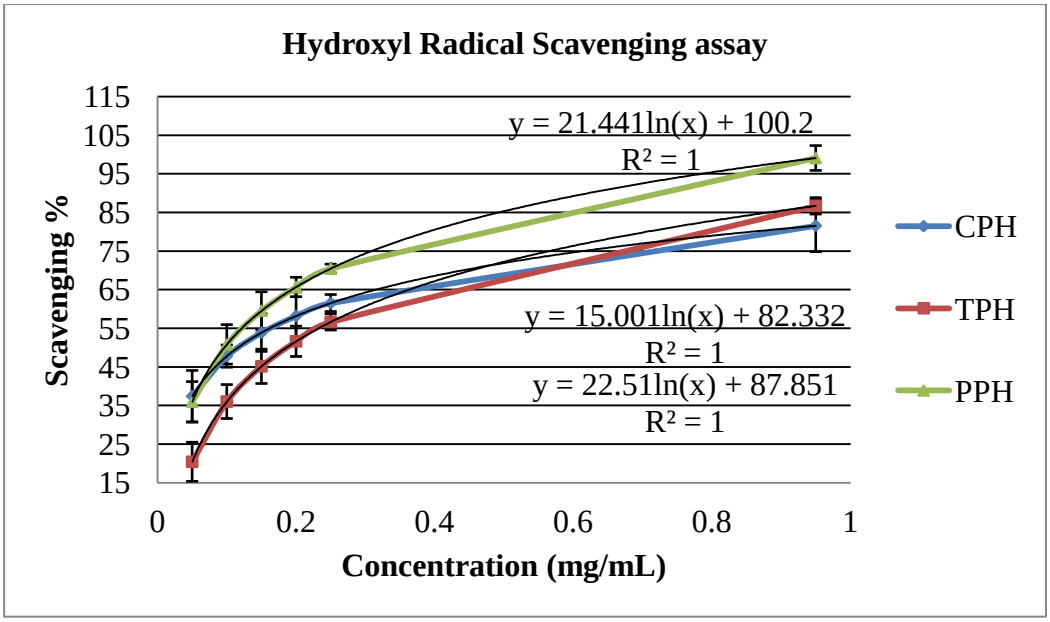

Figure: 4.4. Hydroxyl radical scavenging capacity of CPH, TPH and PPH. Values represent the mean ± SD of triplicates.

The relationship between antioxidant concentration and activity was found to be linear-logarithmic (CPH: y = 15ln(x) + 82.33; PPH: y = 21.44ln(x) + 100.2, TPH; y = 22.51ln(x) + 87.85). The trypsin-treated sample had an IC_{50} value of 0.186 mg/mL and the highest of the three. This indicates it was the poorest antioxidant. According to the study done by Yang, Ke, Hong, Zeng, and Cao (2011), a low IC_{50} value translates into a higher antioxidant activity. The α-Chymotrypsin treated sample (CPH) had the second highest IC_{50} value of 0.116 mg/mL, and the PPH sample had the highest radical scavenging capacity based on its IC_{50} value of 0.096 mg/mL.

In other words, PPH require the least concentration to attain a percent scavenging activity similar to the other peptides. For example, at 0.25 mg/mL PPH reach a scavenging activity of 70.48%, whereas α-chymotrypsin-treated samples and trypsin-hydrolyzed samples reach 61.54% and 56.64% activity, respectively. Thus, in comparison with the standard sample, ascorbic acid, which had an IC_{50} of 0.442 mg/mL, a relatively lower concentration of dogfish skin protein hydrolysates is required to achieve the same extent of antioxidant activity as the standard.

The study shows that the dog-fish derived protein hydrolysates have a hydroxyl radical scavenging activity that is much higher than that of the tuna head protein hydrolysate (THPH), studied by Yang et al., (Yang, Ke, Hong, Zeng, & Cao, 2011), and the peptides from grass carp muscle (GCMH-I, GCMH-II, GCMH-III, GCMH-IV), studied by Ren, et al. (2008). The THPH activity of 2.5mg/mL<IC50<5.0mg/mL, is roughly 19 x lower than that of all three dog-fish derived peptides together (Yang, Ke, Hong, Zeng, & Cao, 2011). The grass carp muscle peptide with the highest hydroxyl radical scavenging activity, IC_{50} = 1.68 ± 0.34 mg/mL, is roughly 13x lower in activity CPH (Ren, et al., 2008). At 3 mg/mL, the big eye tuna dark muscle protein hydrolysates produced by α-chymotrypsin and trypsin had hydroxyl radical scavenging activity less than 50% , whereas papain treated sample was close to 80% in their hydroxyl radical scavenging ability (Je, Qian, Lee, Byun, & Kim, 2008).

Paired t test statistical analysis of the data obtained with the hydroxyl radical scavenging capacity shows PPH is significantly different from CPH ($P<0.0459$) and TPH ($P<0.0001$), while the TPH action profile is significantly similar to CPH ($P<0.0613$).

4.3.3. Superoxide Anion scavenging assay:

Superoxide anions are highly active substances that attack compounds, such as pyrogallol, causing its autooxidation. In this assay the autooxidation of pyrogallol in basic media by superoxide anion is used as a standard against which the activity of standard antioxidant GSH and dog-fish derived protein hydrolysates are measured (Yang, Ke, Hong, Zeng, & Cao, 2011).

Pyrogallol autooxidises in basic media and the buffers used in the assay are all relatively basic. Pyrogallol breaks down into superoxide anion radical and other chromophoric end products; the radical then attacks another pyrogallol molecule, creating more radicals (Yang, Ke, Hong, Zeng, & Cao, 2011). By scavenging this radical the rate of pyrogallol autooxidation can be significantly reduced. The end products of pyrogallol are measured spectrophotometrically at 325 nm. The higher the absorbance, the more end products are present, which means the lower the scavenging activity there is (Yang, Ke, Hong, Zeng, & Cao, 2011).

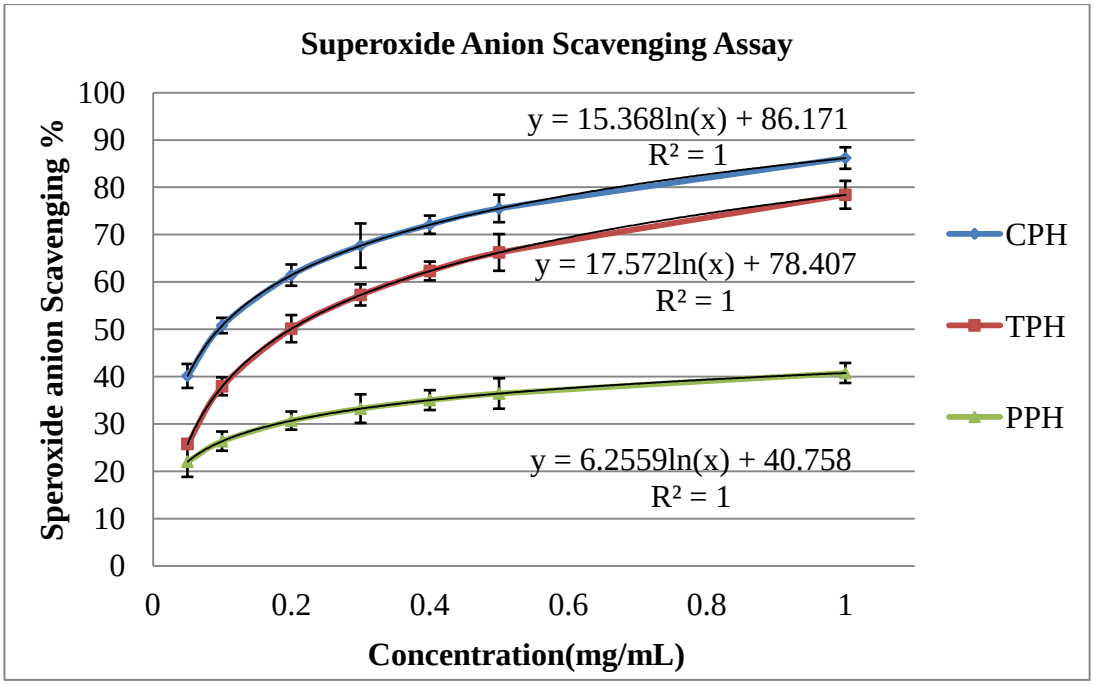

Figure: 4.5. Superoxide anion scavenging capacity of CPH, TPH and PPH. Values represent the mean ± SD of triplicates.

According to the results above, there is a linear-logarithmic relationship between percent inhibition (antioxidant activity) and the concentration of the antioxidant (CPH: y = 15.36ln(x) + 86.17; PPH: y = 6.255ln(x) + 40.75; TPH: y = 17.57ln(x) + 78.4]. CPH and TPH seem to have a relatively similar inhibition activity. However, PPH demonstrates the least superoxide anion radical scavenging activity, with an IC_{50} of 4.388 mg/mL, compared to the IC_{50} of CPH (0.095 mg/mL), TPH (0.198 mg/mL) and GSH (0.0156 mg/mL). The maximum inhibition activity reached by 0.02 mg/mL GSH is 98.35%. For the same percent activity approximately 2.21 mg/mL CPH will be required.

With a higher concentration of protein hydrolysate than GSH, the same efficiency of inhibition can be reached. This shows significant antioxidant property, especially superoxide anion radical scavenging of dog-fish derived protein hydrolysates. The scavenging activity obtained with of 0.60 g/L wheat germ protein hydrolysates against superoxide radical is 75.40% (Y.-h. Cheng, Wang, & Xu, 2006). Research on bigeye tuna dark muscle protein by Je et al., (Je, Qian, Lee, Byun, & Kim, 2008) showed that 3 mg/mL of protein hydrolysates produced by α-chymotrypsin and trypsin was less than 5%, but papain treated sample had more than 20% scavenging activity. Unlike the results obtained by Je at al., (2008), our research with α-chymotrypsin and trypsin treated

dogfish skin protein hydrolysates achieved higher scavenging capacity than their papain treated sample. Also compared to bigeye tuna dark muscle protein hydrolysates, dogfish skin protein hydrolysate had a higher scavenging capacity at 3 mg/ml concentration.

Statistical comparison of the activity profiles of the superoxide anion scavenging capacity of the protein hydrolysates produced by the different enzymes shows that the effects were significantly different from each other ($P<0.05$).

4.3.4. Metal ion chelating Assay:

In this assay, ferrozine combines with ferrous ion to form a purple colored complex, which is measured at 562 nm (Dinis, Madeira, & Almeida, 1994). In this study, dogfish-skin-derived protein hydrolysates produced via α-chymotrypsin, papain, or trypsin-hydrolysis were investigated for ferrous ion chelating activity. This activity was compared with the standard EDTA chelating effect (%).

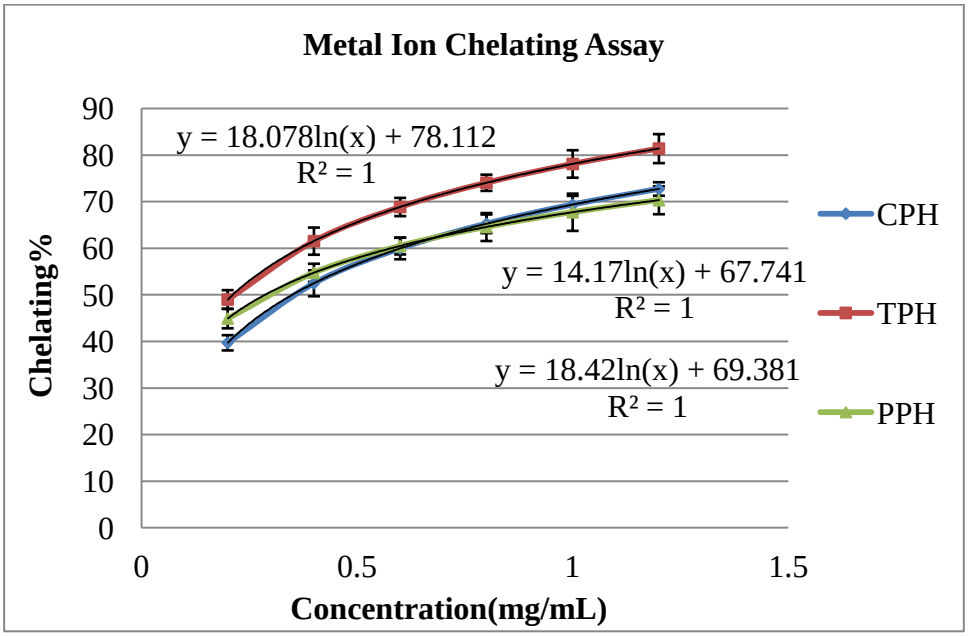

Figure: 4.6. Metal ion chelating capacity of CPH, TPH and PPH. Values represent the mean ± SD of triplicates.

According to the results shown in Figure 4.6 all the three protein hydrolysates showed significant chelating activity. Percent chelating effect had a linear-logarithmic relation with the concentration of the CPH: $y = 18.42\ln(x) + 69.38$; PPH: $y = 14.17\ln(x) + 67.74$; and TPH: $y = 18.07\ln(x) + 78.11$). In other words, the protein hydrolysates chelating

activity increased with increasing concentration until a maximum 100% activity, after which the activity levelled off irrespective of further increases in the protein hydrolysates concentration.

The three protein hydrolysates differed from one another in the extent of chelating activity. At a concentration of 1 mg/mL, TPH had 78.11% chelating affect, while CPH and PPH had only 69.58% and 67.74% chelating effects, respectively. Also, the IC_{50} value of TPH was the lowest (i.e., 0.211 mg/mL), compared to CPH (0.349 mg/mL) and PPH (0.286 mg/mL). The statistical comparison of the metal ion chelating profiles indicated that the effects by CPH and PPH were not significantly different ($P<0.6585$) while the effect by TPH was significantly different from CPH ($P<0.0001$) and PPH ($P< 0.0005$) in their way of chelating the ferrous ion.

The reference chelating agent EDTA had an IC_{50} of 1.898μg/mL, which is much lower than those of the dogfish skin protein hydrolysates. This shows that much more of the protein hydrolysates would be needed to attain the same level of metal ion chelating efficiency as EDTA. The chelating assay involved concentrations of up to 2.72 mg/mL for TPH, for which a maximum chelating activity of 99.82% was observed. Singh and Rajini (2004) studied the antioxidant activities of potato peel extract (PPE), which gave result of IC_{50} of 5 mg/ml for metal chelation, roughly 17x time lower than all three protein hydrolysates from dog fish amounts . In a study by Dong et al. (Dong, Zeng, Wang, Liu, Zhao, & Yang, 2008), with silver crap protein hydrolysates produced by alcalase, it was found that 5 mg/mL of the hydrolysate produced after 4 h treatment had over 80% metal chelating activity, whereas the flavourzyme derived counterpart has less than 70% chelating activity.

4.4. Enzyme Inhibitor Activity:

4.4.1. Lipase Inhibitor Activity:

The protein hydrolysates produced by α-chymotrypsin, papain, and trypsin digestion of the defatted dogfish skin were investigated for lipase inhibition capacity. From the figure 4.7 it was found that at 0.35 mg/mL concentration, the CPH inhibited porcine pancreatic

lipase activity by 69.8%. Instead of the expected lipase enzyme inhibiting activity, TPH and PPH seemed to increase the porcine lipase activity by 1.35x and 1.15x.

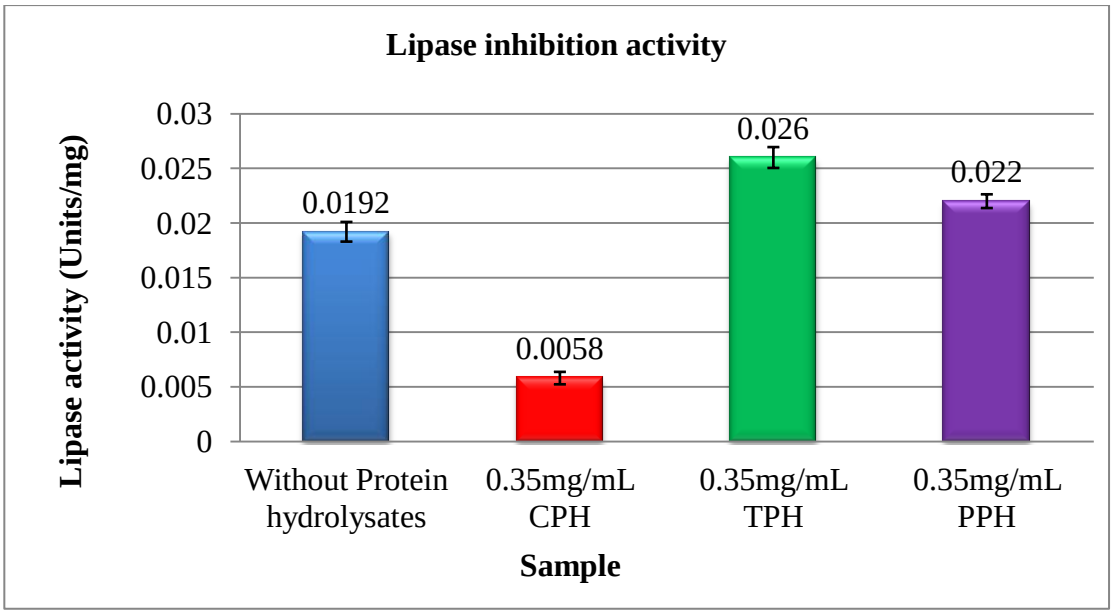

Figure: 4.7. Effect of CPH, TPH and PPH on porcine lipase activity. Values represent the mean ± SD of triplicates.

A study by Bitou et al., (Bitou, Ninomiya, Tsujita, & Okuda, 1999) found an inhibitor, caulerpenyne, from the marine algae (*Caulerpa taxifolia*), that inhibited pancreatic lipase by 50% with triolein as substrate. Nonetheless, there is paucity in information in the literature on the inhibitory effects of protein hydrolysates on lipase activity. Thus further investigation of lipase inhibitory effects of CPH would help to fill this gap. The TPH and PPH activity profile is different from CPH, as they cause enhancement of lipase activity; further study as to be done to understand the factors responsible for this difference in activity among hydrolysates.

4.4.2. α-Chymotrypsin Inhibitor Activity:

The protein hydrolysates from the defatted dogfish protein powder were investigated for the α-chymotrypsin inhibitory activity. From figure 4.8 it is found all the three hydrolysates possessed α-chymotrypsin inhibition ability.

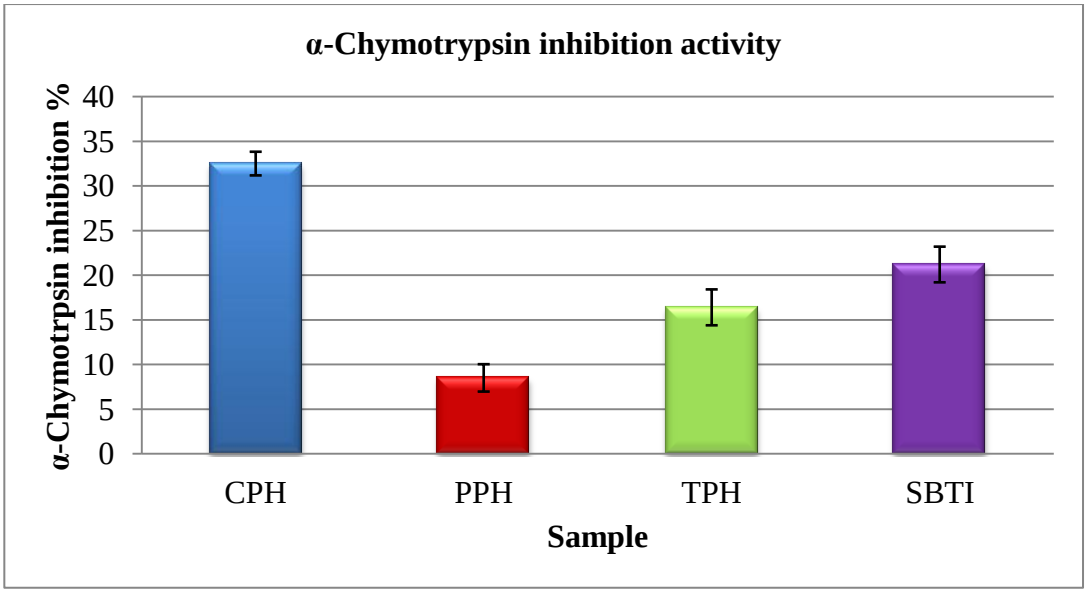

Figure: 4.8. Comparison of α-Chymotrypsin inhibitor activity at 40µg/mL. Values represent the mean ± SD of triplicates.

At a concentration of 40µg/mL, CPH inhibited α-chymotrypsin 32.5% while PPH and TPH inhibited the α-chymotrypsin by 8.5% and 16.4%, respectively. The commercial soybean trypsin inhibitor (40µg/mL) used as the control, inhibited the α-chymotrypsin by 21.2% inhibition.

Hitherto, there is a dearth in the literature on the inhibitory effects of protein hydrolysates on enzyme protease (e.g., α-chymotrypsin) activity, and further investigation of the protease inhibitory effects by the defatted dogfish skin hydrolysates will help to fill this void.

4.5. Antimicrobial activity:

The defatted dogfish skin hydrolysates produced with the 3 enzymes, i.e., CPH, TPH and PPH were investigated for antimicrobial activity using two different concentration (i.e., 2 and 12 mg/mL) against three microbial strains, namely: DH5α *E.coli*, *Bacillus subtilis* and *Lactococcus lactis*. The hydrolysates were filtered using 0.22µM pore size filter to free them from microbial contamination.

4.5.1. Disc Diffusion Method:

From table 4.2 below, it is found that in the study for antimicrobial activity, all the three protein hydrolysates were incapable of inhibiting the growth of the targeted microorganisms at the 2 levels investigated (i.e., 2 vs 12 mg/mL). This inactivity towards the targeted microorganisms could be either due to insensitivity by this particular set of tested microorganisms for the protein hydrolysates, or the absence of peptides with antimicrobial activities in the extract. It may also be due to inadequate exposure time for the hydrolysate to interact with the microorganisms. Thus, further studies with other microorganisms or the purified peptides at different concentrations would help to clarify this uncertainty. The polymyxin B used as a positive control demonstrated antimicrobial activity against DH5α *E.coli* and *Bacillus subtilis*, but not *Lactococcus lactis.*

Table: 4.2. Study on antimicrobial activity by disc diffusion method:

Sample	**DH5α *E.coli***	***Bacillus subtilis***	***Lactococcus lactis***
2mg/mL of CPH	+	+	+
2mg/mL of TPH	+	+	+
2mg/mL of PPH	+	+	+
12mg/mL of CPH	+	+	+
12mg/mL of TPH	+	+	+
12mg/mL of PPH	+	+	+
POLYMYXIN B	-	-	+

+ growth of microbial cell were observed (no inhibition zone), - inhibition zone were observed

4.5.2. Survival Assay:

When the three microorganisms were subjected to different incubation times, the following observations were made:

4.5.2.1. Effect of Protein Hydrolysates Concentration on the growth of DH5α *E.coli* strain:

As shown in Figure 4.9 below, when 2 mg/mL of CPH was applied to the DH5α- *E.coli* strain, there was little decrease in growth with increasing incubation time from 0.5 h to 4 h. Growth increased up to 48 h but had declined after 96 h. A similar pattern was observed with TPH at the 2 mg/mL level, there was increase in growth from 4 h to 48 h, but had declined after 96 h. With PPH at the 2 mg/mL level, the growth of DH5α- *E.coli* strain appeared to remain steady from 0.5 h to 48 h of incubation after which there was a slight increase in CFU at 96 h.

The following observations were made with CPH and TPH on *DH5α- E.coli strain* at the 12 mg/mL level; growth decreased from 0.5 h to 4 h and appeared to remain steady up to 48 h and beyond to 96 h. 12 mg/mL of PPH incubated microbial cells found to have slight decrease at 4 h (7.8E+0.7 CFU) than 0.5 h (7.9E+07 CFU), but resulted with increase in growth of microbial cells at 48 h (8.0E+07 CFU) and 96 h (8.20E+07 CFU). Polymyxin B which was used as a positive control showed no growth of microbial cells. When sterile deionised water was substituted instead of protein hydrolysates the growth was steady from 0.5 h to 96 h with CFU of 6.8 E+07.

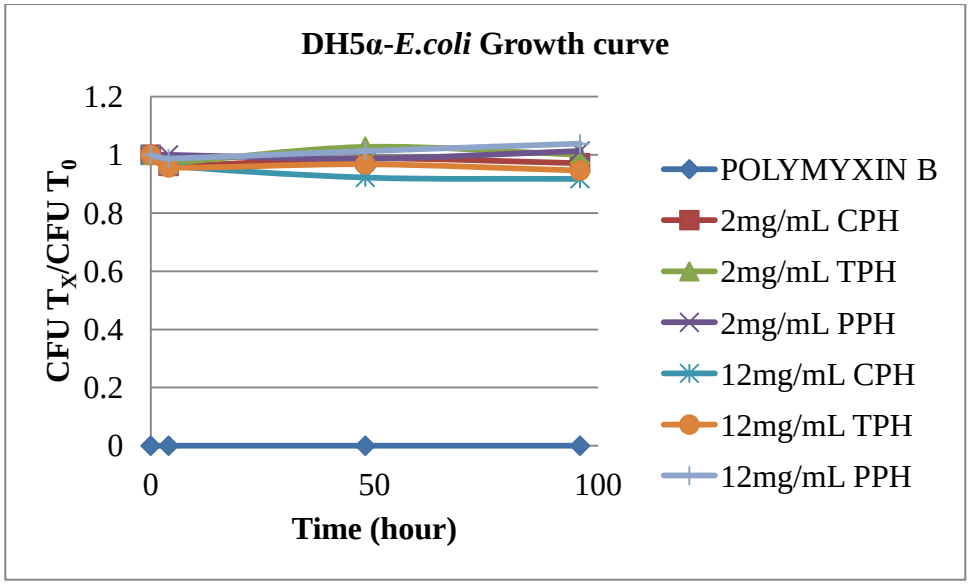

Figure: 4.9. Effect of different protein hydrolysates concentration on the growth of DH5α *E.coli* strain.

Table 4.3 below, shows the P values of a paired t test based statistical study done on comparing the activity profile of three hydrolysates against DH5α- *E.coli* strain at two levels (2 and 12 mg/mL). From table 4.3 it is found that all trial found to have a significantly similar profile of activity against the DH5α- *E.coli* strain.

Table: 4.3. P values from the paired t test based on the effects of protein hydrolysates on the growth of DH5α- *E.coli* strain.

Trial	Pairs compared	Prob > |t|
1	2 mg/mL CPH vs 2 mg/mL TPH	0.1162
2	2 mg/mL TPH vs 2 mg/mL PPH	1
3	2 mg/mL PPH vs 2 mg/mL CPH	0.1817
4	12 mg/mL CPH vs 12 mg/mL TPH	0.201
5	12 mg/mL TPH vs 12 mg/mL PPH	0.1221
6	12 mg/mL PPH vs 12 mg/mL CPH	0.1162
7	2 mg/mL CPH vs 12 mg/mL CPH	0.1904
8	2 mg/mL TPH vs 12 mg/mL TPH	0.1343

9	2 mg/mL PPH vs 12 mg/mL PPH	0.3534
10	2 mg/mL CPH vs 12 mg/mL TPH	0.1817
11	2 mg/mL TPH vs 12 mg/mL PPH	0.495
12	2 mg/mL PPH vs 12 mg/mL CPH	0.0837
13	2 mg/mL CPH vs 12 mg/mL PPH	0.1343
14	2 mg/mL TPH vs 12 mg/mL CPH	0.1586
15	2 mg/mL PPH vs 12 mg/mL TPH	0.1027

4.5.2.2. Effect of Protein Hydrolysates Concentration on the growth of *Bacillus subtilis* strain:

Bacillus subtilis works as a probiotic in healthy individuals with resistance to extreme stress like UV, heat, high pressure, acidity etc. In this study, the three protein hydrolysates (at 2 mg/mL and 12 mg/mL levels) were investigated for their effects on the growth of *B. subtilis* on nutrient agar medium.

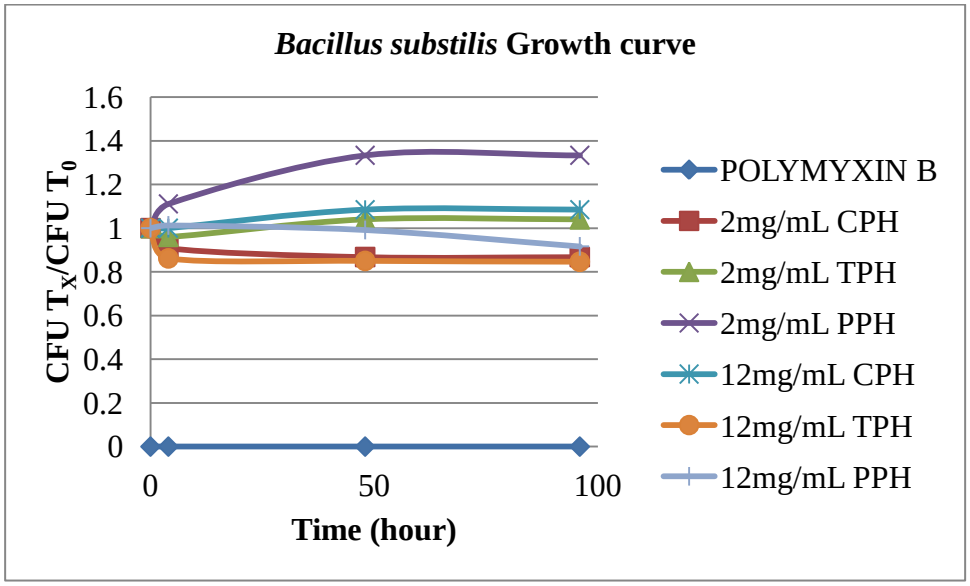

Figure: 4.10. Effect of different protein hydrolysates concentration on the growth of *Bacillus subtilis* strain

As shown in figure 4.10, when 2 mg/mL of CPH was applied to *B.subtilis* strain, there was decrease in growth of microbial cells with increase in incubation time from 0.5 h to 4

h, further increase in incubation up to 48 h shows slight decrease in growth which appeared to remain steady beyond 96 h. With TPH at 2 mg/mL level, the growth of *B.subtilis* decrease from 0.5 h to 4 h of incubation, but increase in incubation up to 48 h had slight increase in growth of the microbial cells which appeared to remain steady up to 96 h. *B.substilis* strain incubated with PPH at 2mg/mL level shows increase in growth of microbial cell from 0.5 h to 4 h; with extended incubation up to 48 h observed to have slight increase in growth which appeared to be steady when incubated till 96 h.

The following observation were made with CPH on *B.subtilis* at 12 mg/mL level; growth of microbial cells were steady up to 4 h; with increase in incubation up to 48 h shows the growth increases which appeared to become steady with further incubation to 96 h. With TPH at 12mg/mL level on *B.subtilis* strain, growth increased with increase in incubation from 0.5 h up to 4 h, further increase in incubation appeared to have steady growth up to 96 h. Incubation of *B.subtilis* with 12 mg/mL of PPH shows steady growth till 48 h, slight decrease in growth is observed with further incubation till 96 h. Effect of Polymyxin B was checked as a positive control which actively inhibited the growth of *Bacillus subtilis* strain completely. With deionised water as negative control against *Bacillus subtilis* growth, observed to have a steady growth up to 96h (CFU value range of 2E+06). But when this trial was compared to growth of *Bacillus subtilis* in presence of protein hydrolysates it seems to be decreasing slightly.

Table: 4.4. P values from the paired t test based on the effects of protein hydrolysates on the growth of *Bacillus subtilis* strain.

Trial	Pairs compared	Prob > |t|
1	2 mg/mL CPH vs 2 mg/mL TPH	0.11
2	2 mg/mL TPH vs 2 mg/mL PPH	0.08
3	2 mg/mL PPH vs 2 mg/mL CPH	0.09
4	12 mg/mL CPH vs 12 mg/mL TPH	0.07
5	12 mg/mL TPH vs 12 mg/mL PPH	0.08
6	12 mg/mL PPH vs 12 mg/mL CPH	0.23
7	2 mg/mL CPH vs 12 mg/mL CPH	0.09

8	2 mg/mL TPH vs 12 mg/mL TPH	0.08
9	2 mg/mL PPH vs 12 mg/mL PPH	0.12
10	2 mg/mL CPH vs 12 mg/mL TPH	0.12
11	2 mg/mL TPH vs 12 mg/mL PPH	0.47
12	2 mg/mL PPH vs 12 mg/mL CPH	0.09
13	2 mg/mL CPH vs 12 mg/mL PPH	0.09
14	2 mg/mL TPH vs 12 mg/mL CPH	0.06
15	2 mg/mL PPH vs 12 mg/mL TPH	0.08

Table 4.4 shows the P values of a paired t test based statistical study, which is done on comparing the activity profile of three hydrolysates against *B.subtilis* strain at two levels (2 and 12 mg/mL). From table 4.4 it is found, all trials are not significantly different in their profile of activity against *B.subtilis* strain.

4.5.2.3. Effect of Protein Hydrolysates Concentration on the growth of *Lactococcus lactis* strain:

Lactococcus lactis is one of the most important and better known probiotic bacteria and used in many dairy fermentation products. The results of the effects of different concentrations of the protein hydrolysates on the growth of *Lactococcus lactis is presented* in Figure: 4.11.

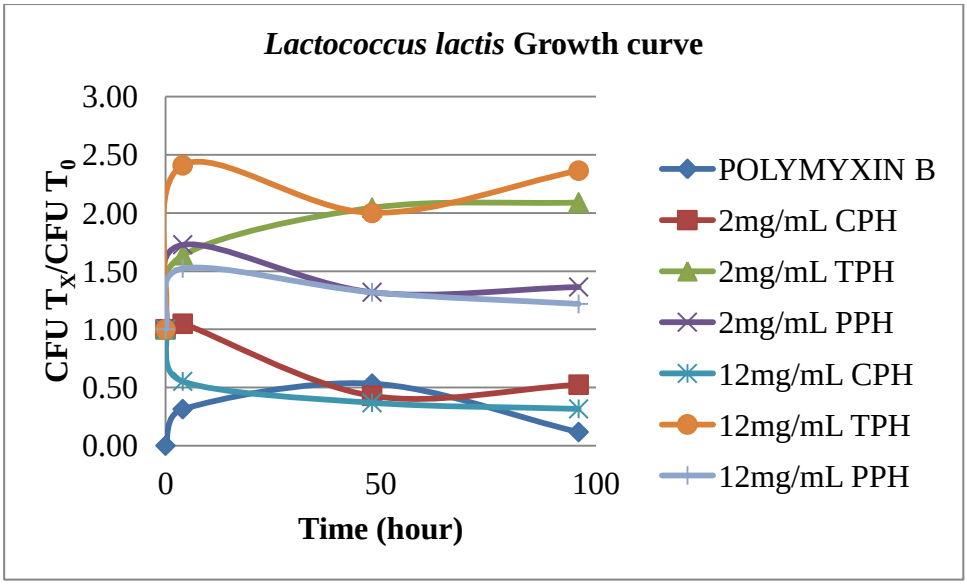

Figure: 4.11.Effect of different protein hydrolysate concentrations on the growth of *Lactococcus lactis* strain

As presented in figure 4.11, CPH on *Lactococcus lactis* strain at 2mg/mL level; growth slightly increased till 4 h of incubation, with further incubation the growth of organisms decreased up to 48 h and appeared to increase with incubation beyond 96h. A similar pattern was observed with PPH on *Lactococcus lactis* strain at the 2 mg/mL level, there was increase in growth from 0.5 h to 4 h, but declined at 48 h and further increased in growth after 96 h incubation. With TPH at 2mg/mL as shown increase in growth from 0.5 hour incubation to 48 hour, where with further incubation up to 96 h appeared to have steady growth of microbial cells.

The following observations were made with CPH at 12mg/mL on *Lactococcus lactis* strain, growth declined with increase in incubation from 0.5 h to 4 h and appeared to be steady up to 48 h and beyond 96 h. With TPH at 12mg/mL level, the growth of *Lactococcus lactis* cell increased by 2x from 0.5h to 4h, but slight decrease with increase in incubation till 48hour and increase beyond 96 h incubation. PPH at 12mg/mL have a similar pattern like PPH at 2mg/mL on *Lactococcus lactis* strain; growth increase with increase in incubation from 0.5 h to 4 h, with decline growth of microbial cells up to 48 h and appeared to increase beyond 96 h.

From figure 4.11 it was observed *Lactococcus lactis* strain growth was not inhibited completely by polymxyin B as compared to DH5α *E.coli* and *B.subtilis* strain. Growth declined by 2.5x with increase in incubation from 0.5 h to 4 h, but with increase in incubation up to 48 h increased the growth of the microbial cells and further continued to decrease in growth when incubated beyond 96 h. *L.lactis* incubated with deionised water (negative control) had less growth (6.00E+07 to 8.00E+07 CFU) compared to the growth in presence of protein hydrolysates.

Table: 4.5. P values from the paired t test based on the effects of protein hydrolysates on the growth of *Lactococcus lactis* strain.

Trial	Pairs compared	Prob > |t|
1	2 mg/mL CPH vs 2 mg/mL TPH	0.10
2	2 mg/mL TPH vs 2 mg/mL PPH	0.22
3	2 mg/mL PPH vs 2 mg/mL CPH	0.06
4	12 mg/mL CPH vs 12 mg/mL TPH	0.06
5	12 mg/mL TPH vs 12 mg/mL PPH	0.07
6	12 mg/mL PPH vs 12 mg/mL CPH	0.06
7	2 mg/mL CPH vs 12 mg/mL CPH	0.19
8	2 mg/mL TPH vs 12 mg/mL TPH	0.28
9	2 mg/mL PPH vs 12 mg/mL PPH	0.19
10	2 mg/mL CPH vs 12 mg/mL TPH	0.06
11	2 mg/mL TPH vs 12 mg/mL PPH	0.14
12	2 mg/mL PPH vs 12 mg/mL CPH	0.06
13	2 mg/mL CPH vs 12 mg/mL PPH	0.07
14	2 mg/mL TPH vs 12 mg/mL CPH	0.07
15	2 mg/mL PPH vs 12 mg/mL TPH	0.07

Table 4.5 shows the P values of a paired t test based statistical study, which is done on comparing the activity profile of three hydrolysates against *Lactococcus lactis* strain at

two levels (2 and 12 mg/mL). From table 4.5 it is observed all the trials have statistically no significant difference in their activity profile against *L. lactis* strain.

4.5.3. Discussion on factors affecting of antimicrobial activity:

From the experiments on the antimicrobial effects of the defatted dogfish skin hydrolysates against the three different bacterial strains, it is observed that there were no great variations in the increase or decrease in the growth of microbial cells by the three different protein hydrolysates. The microbial growth profiles as evidenced by the CFU values were quite similar both in numbers and trends. The hydrolysates also did not appear to have antimicrobial capacity; however from the preliminary results obtained from the *B. subtilis* and *L.lactis* studies, it appears that the protein hydrolysates could enhance the survival of these two probiotic bacteria, which could be of significant benefit to the beverage and nutraceutical industries.

There are other factors that can impact antimicrobial activities. A review by Ramos-Villarroel et al., (Ramos-Villarroel, Soliva-Fortuny, & Martín-Belloso, 2010) indicates that factors such as hydrophobicity, pH, ionic strength, temperature, surfactants, among others, can all affect or alter antimicrobial activity. Branen and Davidson (2000) reported that the addition of EDTA could enhance the antimicrobial activity of pepsin treated lactoferrin hydrolysate. Thus, investigating the defatted dogfish skin hydrolysis in combination with some of these combinations could provide new information from what is reported here.

Chapter 5: CONCLUSION AND RECOMMENDATION

5.1. Conclusion:

From the data presented in the results and discussion sections, the following conclusions are made.

1. Hydrolysis of defatted dogfish skin by 1% α-chymotrypsin for 4 h gave the most antioxidant activity based on the DPPH assay, and this enzyme concentration and time of hydrolysis were used for the subsequent hydrolysis with the other enzymes.
2. Ultracentrifugal filtration of the protein hydrolysates had practically no residual enzyme in the filtrates and this method was used to rid the hydrolysates of residual enzyme activity after the desired hydrolyses were attained.
3. For antioxidant activities by the hydrolysates, PPH had highest reducing power and hydroxyl radical scavenging ability compared to TPH and CPH. CPH had lowest IC_{50} value of 0.095mg/mL for scavenging superoxide radical when compared to TPH (0.199mg/mL) and PPH (4.388mg/mL). Metal chelating activity by TPH was highest compared to PPH (by 1.34x) and CPH (1.79x).
4. For the enzyme inhibitory activities by the hydrolysates, only CPH had lipase inhibitory activity, and inhibited porcine lipase activity by 69.8%. The TPH and PPH seemed to rather enhance the porcine lipase activity by 1.35x and 1.15x, respectively. CPH had highest bovine pancreatic α-Chymotrypsin inhibitory activity and achieved 32.5% inhibition of the enzyme at 40μg/mL compared to TPH (which inhibited the enzyme by 16.4 % at 40μg/mL), PPH (which inhibited the enzyme by 8.5 % at 40μg/mL) and commercial soybean trypsin inhibitor (which inhibited the enzyme by 21.2% at 40μg/mL).
5. For the antimicrobial effects, the hydrolysates appeared to have no antimicrobial capacity against DH5α *E.coli*, *Bacillus subtilis* and *Lactococcus lactis.* The protein hydrolysates rather appeared to enhance the survival of the two probiotic bacteria (*B. subtilis* and *L. lactis*) that were investigated.
6. Overall, this study discovered that CPH had more potent bioactive effects in terms of antioxidant and enzyme inhibitory effects than the PPH and TPH.

5.2. Recommendations for further work:

The following recommendations are made for future research on CPH:

i. purification and biochemical characterization of the peptides in the fish skin hydrolysates;
ii. biological activity testing of the purified peptides;
iii. selection of the most promising peptides for sequencing and scaled up production;
iv. testing of the most bioactive peptides in model food systems.

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