PACIFIC HAKE (*Merluccius productus*) FISH PROTEIN HYDROLYSATES WITH ANTIOXIDATIVE PROPERTIES

by

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Abstract

Pacific hake (*Merluccius productus*) is an under-valued fish with high endogenous proteolytic activity due to parasitic infection by *Kudoa paniformis*. The objective of this research was to produce fish protein hydrolysates (FPH) with antioxidative properties from Pacific hake and to assess their potential to inhibit oxidative processes, *in vivo*. Proteolytic activity of fish homogenate was optimum at pH 5.25-5.50 and 52-55°C. Significant ($p<0.05$) correlations were observed among fish mince *K. paniformis* spore counts, endogenous proteolytic activity, and content of free amino groups during autolysis. Autolysis of *Kudoa*-infected fish fillet mince (30×10^6 spores/g) for 1 h at 52 °C and pH 5.50 produced FPH with high antioxidative capacity in a linoleic acid model system, which was higher ($p<0.05$) than that of butylated hydroxyanisole and α-tocopherol over prolonged storage (~162 hrs). Trolox equivalent antioxidant capacity and oxygen radical absorbing capacity values of FPH were 262 ±2 and 225 ±17 μmol Trolox equivalents/g freeze-dried sample, respectively. Further, FPH could scavenge 1,1-diphenyl-2-picrylhydrazyl radicals, but iron-chelating ability was very low. When *in vitro* angiotensin-I-converting enzyme (ACE)-inhibitory potential was quantified to examine potential multifunctional capacity, FPH was shown to be a substrate-type inhibitor of ACE with IC_{50} of 162 μg peptides/mL. *In vitro* gastrointestinal (GI)-digestion of FPH increased ($P<0.05$) antioxidative capacity, while ACE-inhibitory activity remained unchanged. Some peptides from FPH and GI-digested FPH passed through Caco-2 cells, and the permeates showed 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging capacity but not ACE-inhibitory activity. FPH and GI-digested FPH inhibited 2,2′-azobis-(2-amidinopropane) dihydrochloride-induced oxidation in Caco-2 cells at non-cytotoxic concentrations. Both antioxidative and ACE-inhibitory peptides were concentrated in a 1-3 kDa fraction by ultrafiltration, but the peptides having antioxidative capacity differed from those exhibiting ACE-inhibitory activity. Two peptides (PLFQDKLAHAK and AEAQKQLR) were identified and their antioxidative potential, along with three other literature reported antioxidative peptides, were assessed in different *in vitro* chemical systems; antioxidative capacities depended on peptide sequence, amino acid composition, and the *in vitro* assay system used.

In conclusion, antioxidative FPH from Pacific hake could be a useful functional food ingredient. Further studies are needed to assess prospective antihypertensive effects and to identify antioxidative mechanisms of constituent peptides.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AAPH	2,2′-azobis-(2-amidinopropane) dihydrochloride
ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
ACE	Angiotensin-I-converting enzyme
AUC	Area under the curve
BHA	Butylated hydroxanisole
BHT	Butylated hydroxytoluene
C-(1-2)h	Control protein hydrolysate made with Pacific hake fish fillet mince by (1-2) h incubation at pH 5.50 and 52ºC after inactivating endogenous enzymes
Caco-2	Human adenocarcinoma colon cancer
DCFH-DA	2′,7′-dichlorofluorescin diacetate
DH	Degree of hydrolysis
DPPH	2,2-diphenyl-1-picrylhydrazyl
DMEM	Dulbecco's modified eagle's medium
E-(1-6) h	Hydrolysate made with (1-6) h autolysis of Pacific hake fish fillet mince at pH 5.50 and 52ºC
EDTA	Ethylenediamine tetraacetic acid
ET	Electron transfer
EPA	Eicosapentaenoic acid
ESR	Electron spin resonance
F-(1-2) h	Hydrolysate made with Flavourzyme™ by (1-2) h hydrolysis at pH 5.50 and 52ºC
FCR	Folin-Ciocalteu reagent
FDA	Food and drug administration
FPH	Fish protein hydrolysate
GC	Gas chromatography
GF-FPLC	Gel filtration fast protein liquid chromatography
GI	Gastro-intestinal
HAT	Hydrogen atom transfer

HBSS	Hank's balanced salt solution
HHL	Hippuryl-histidyl-leucine tetrahydrate
IC_{50}	Inhibitory concentration 50 (concentration of inhibitory agent required to inhibit the activity by 50 %)
Inf-(0-100)	Pacific hake fish mince with *K. paniformis* infection level of (0-100) x 10^6 spores/g mince
IMAC	Immobilized metal affinity chromatography
IOU	Inhibited oxygen uptake
LC-MS/MS	Liquid chromatography with tandem mass spectrometry detection
LDL	Low-density lipoprotein
MALDI-ToF	Matrix-assisted laser desorption/ionization time of flight
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ORAC	Oxygen radical absorbance capacity
PB	Phosphate buffer
RMCD	Randomly methylated β-cyclodextrin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP-HPLC	Reversed-phase high performance liquid chromatography
RSM	Response surface methodology
SHR	Spontaneously hypertensive rat
TBARS	2-thiobarbituric acid reactive substances
TEAC	Trolox equivalent antioxidant capacity
TEER	Transepithelial electrical resistance
TFA	Trifluoroacetic acid
TNBS	2,4,6-trinitrobenzenesulfonic acid
TOSC	Total oxidant scavenging capacity
TRAP	Total radical-trapping antioxidant parameter
UF	Ultrafiltration
V-(1-2) h	Hydrolysate made with Validase® BNP by (1-2) h hydrolysis at pH 5.50 and 52ºC

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To my loving parents and husband
who were always there for me in every step of the way
to come this far…

Co-Authorship Statement

The work presented in Chapter 2 of this thesis was published in the Journal of Aquatic Food Product Technology (2006, 15(4): 75-93), entitled "Correlation of *Kudoa* spore counts with proteolytic activity and texture of fish mince from Pacific hake (*Merluccius productus*)". AGP Samaranayaka, the thesis author, was the principal author who performed most of the research, analyzed data, and prepared the manuscript. TCW Ho, a co-author of this study helped in *Kudoa* spore counting of fish mince batches as well as fish tissue samples taken from different locations of the Pacific hake whole fish as described in Chapter 2 methodology section. ECY Li-Chan, AGP Samaranayaka's supervisor, was the other co-author and provided close guidance on performing the above research.

The work presented in Chapter 3 was published in Food Chemistry (2008, 107: 768-776), titled "Autolysis-assisted production of fish protein hydrolysates with antioxidant properties from Pacific hake (*Merluccius productus*)". AGP Samaranayaka, the thesis author was the principal author and ECY Li-Chan, AGP Samaranayaka's supervisor was the co-author.

The work described in Chapter 4 was published in Journal of Agricultural and Food Chemistry (2010, 58: 1535-1542), titled "Antioxidative and angiotensin-I-converting enzyme inhibitory potential of a Pacific hake (*Merluccius productus*) fish protein hydrolysate subjected to simulated gastrointestinal digestion and Caco-2-cell permeation". AGP Samaranayaka, the thesis author was the principal author. ECY Li-Chan, AGP Samaranayaka's supervisor, and DD Kitts, member of AGP Samaranayaka's supervisory committee, co-authored this work and provided guidance on the research and manuscript preparation.

CHAPTER 1 Introduction and Literature Review

1.1 Study Context

The development of fish protein hydrolysates (FPH) and peptides as functional food ingredients is a relatively recent technology gaining in popularity due to the array of potential bioactive properties associated with them, including antioxidant, antihypertensive, immunomodulatory, neuroactive, antimicrobial, and mineral or hormone regulating properties (Alasalvar and others 2002). A substantial amount of information is available on antimicrobial and antihypertensive peptides derived from fish muscle and FPH. An increasing amount of research performed during last decade in identifying antioxidative peptides from various FPH clearly demonstrates the potential of using these peptides in various food and pharmaceutical applications. This research area, however, is relatively new and much work has still to be done in order to identify appropriate enzymes and substrates (i.e. fish byproducts and under utilized fish) to be used to produce FPH with antioxidative properties, to scale-up the processing conditions, and also to identify structure-activity relationships and efficacy of these peptides in biological as well as in food systems.

Pacific hake (*Merluccius productus*) is a low economic value fish because of the presence of a parasitic Myxosporea (genus *Kudoa*) and the "soft flesh" condition that results from autolysis during subsequent processing. However, the decreasing fishing quotas of traditionally harvested species have generated a significant commercial interest in the Pacific hake fish stock (Greer 2002). In 2007, Pacific hake was British Columbia (BC)'s largest single fish species harvest by weight at 74,100 tonnes, which accounted for 41 per cent of the provincial total commercial harvest and 60 percent of the ground fish harvest (The British Columbia Seafood Industry Year in Review 2007). Despite the precautions taken to reduce post mortem quality loss of Pacific hake and despite the introduction of various protease inhibitors such as potato extracts as additives during processing, the likelihood for formation of mushy texture is an ongoing problem that affects the price and market value of this fish. Alternative methods of utilizing this under-valued fish are therefore needed to increase the market value. Due to the increased demand for protein hydrolysates with antioxidative

properties by pharmaceutical, health food, as well as food processing/preservation industries (Alasalvar and others 2002; Hagen and Sandnes 2004), production of FPH possessing antioxidative capacity from Pacific hake muscle proteins and development of FPH-based ingredients for food and pharmaceutical applications will be an ideal approach to utilize this huge stock of Pacific hake caught in waters near BC, Canada.

Investigation on antioxidative components of FPH from Pacific hake and, in particular, the influence of *Kudoa* parasitization on FPH production, have not been conducted so far. Increased endogenous proteolytic activity of *Kudoa*-infected Pacific hake muscle could be an asset, rather than a disadvantage, in making FPH with various functional properties without adding commercial enzymes. The proposed research was, thus, aiming to produce FPH with antioxidative peptides from Pacific hake muscle proteins using endogenous enzymes present in fish muscle and/or with the addition of commercial enzyme preparations. *In vitro* chemical assays were used in assessing the antioxidative potential of this FPH. Since recent research revealed that some of the antioxidative protein hydrolysates also contain peptides possessing other biological activities such as angiotensin-I-converting enzyme (ACE) inhibitory capacity (Davalos and others 2004; Hernandez-Ledesma and others 2005; Nagai and Nagashima 2006; Hernandez-Ledesma and others 2007), Pacific hake FPH was also tested for its ACE-inhibitory potential. Further, potential effect of simulated gastro-intestinal (GI) digestion on these antioxidative and ACE-inhibitory peptides, their bioavailability, bioactivity, and possible toxic effects were assessed using different *in vitro* assays in order to assess the potential to be used as functional food or pharmaceutical ingredients.

1.2 Functional Protein Hydrolysates

Protein hydrolysates obtained by enzymatic or chemical breakdown of food proteins into peptide fragments have long been used for various applications in the food industry including milk replacers, protein supplements, stabilizers in beverages and flavour enhancers in confectionery products (Kristinsson and Rasco 2000). Other uses of protein hydrolysates as described by Mahmoud and Cordle (2000) include as the nitrogen sources for enternal feeding of individuals with specific nutritional or physiological needs, such as infants with intact protein hypersensitivity, or individuals with impaired GI function (e.g. Crohn's

disease), short bowel syndrome or specific organ disease (e.g. pancreatitis, renal and hepatic malfunction), cancer patients and individuals with AIDS. Protein hydrolysates are also used in sports nutrition, weight control diets and nutritional supplements (Mahmoud and Cordle 2000). Above all, recent literature clearly demonstrates the potential use of protein hydrolysates to improve human health due to constituent peptides that can be biologically active (Hartmann and Meisel 2007).

1.3 Biologically Active Peptides

Bioactive peptides refer to specific protein fragments that have a positive impact on body function or condition and which ultimately may influence health beyond their basic role as nutrient sources (Hartmann and Meisel 2007). These peptides are usually short in length (i.e. 3-20 amino acids residues) and their activity is based on size, amino acid composition, and sequence (Kitts and Weiler 2003; Pihlanto and Korhonen 2003). Moreover, the potential to elicit biological responses depends on the ability of the peptides to cross the intestinal epithelium and enter the blood circulation, or to bind directly to specific epithelial cell-surface receptor sites (Pihlanto and Korhonen 2003).

Various food protein sources have been tested as potential sources of peptides which are biologically active. Bioactive peptides may be directly present as such in food, or can be released from different dietary proteins of plant or animal origin during GI digestion or during preparation of protein hydrolysates using exogenous enzymes, food processing or by a fermentation process (Korhonen and Pihlanto 2003). While milk has been by far the most exploited source of bioactive peptides, other major protein sources include egg, animal and fish meat, and various plant sources such as soybean, wheat and maize (Yamamoto and others 2003; Hartmann and Meisel 2007).

1.4 FPH as a Source of Bioactive Peptides

Depending on the type of fishery, by-products or waste generated from seafood processing plants usually accounts for about 30 to 85 % of the weight of the landed catch

(Guérard and others 2005). Due to relatively low cost, these processing discards as well as underutilized aquatic species are valuable sources of raw material for recovery of existing bioactive peptide constituents or for generation of bioactive peptides from the protein components. At the same time, this approach will enhance the market value of low-valued fish and increase the use of seafood processing discards. An array of biological activities have been reported for FPH and specific peptide sequences derived from these hydrolysates (Shahidi 2005; Kim and Mendis 2006; Guérard 2007). These reported bioactivities include antioxidative, antihypertensive, immunomodulatory, neuroactive, antimicrobial, mineral and hormonal regulating properties (Bernet and others 2000; Jun and others 2004; Je and others 2005b; Duarte and others 2006; Jung and others 2006; Je and others 2007; Murray and FitzGerald 2007; Liu and others 2008).

1.4.1 Process of FPH production

Biologically active peptides can be produced from precursor proteins using endogenous proteolytic enzymes already present in muscle or viscera of fish and/or by adding exogenous enzymes from other sources (Kristinsson and Rasco 2000; Kristinsson 2007). Enzymatic hydrolysis has been the most common method of producing bioactive peptides from fish and its by-products (Korhonen and Pihlanto 2003; Guérard 2007). Industrial food-grade proteinases such as Alcalase®, Neutrase®, Flavourzyme®, and Protamex™ derived from microorganisms have been used widely to produce protein hydrolysates with various bioactivities, whereas enzymes from plant (e.g. papain, bromelain, ficin) and animal sources (e.g. pepsin, trypsin) have also been used but to a lesser extent (Guérard 2007). Depending on the raw material used, endogenous enzymes such as trypsin, chymotrypsin, pepsin, other enzymes of viscera and digestive tract, as well as lysosomal proteases or catheptic enzymes in fish contribute to the break down of proteins during autolysis (Kristinsson and Rasco 2000).

Use of exogenous enzymes is preferred in most cases to the autolytic process due to the reduction in time required to achieve similar degree of hydrolysis as well as better control of the hydrolysis to obtain more consistent molecular weight profiles and peptide composition. For example, fish protein hydrolysates with ACE-inhibitory activity (IC_{50} 165 μg/mL) could be consistently prepared using different batches of Pacific hake (*Merluccius productus*) fish

fillet by using exogenous enzyme Protamex^{TM} (Cinq-Mars and Li-Chan 2007; Cinq-Mars and others 2008). In comparison, seasonal, gender, and age dependent changes may lead to variability in endogenous enzymes present in fish and other marine sources (Kristinsson and Rasco 2000; Guérard 2007). Despite the potential problems arising from this variability, endogenous enzymes have been reported in the literature for production of various protein hydrolysates with bioactive peptides. For example, peptides with antioxidative properties could be prepared from Alaska pollack (*Theragra chalcogramma*) and yellowfin sole (*Limanda aspera*) frame proteins by using a crude enzyme mixture from mackerel intestine (Jun and others 2004; Je and others 2005c).

The main steps involved in producing FPH through autolysis or by adding exogenous enzymes are outlined in Fig. 1.1. Underutilized fish or fish by-products are first homogenized and then suspended in water before adjusting pH and/or temperature to initiate the hydrolysis process using autolysis or by adding an exogenous enzyme. In some cases, the raw material is first heated to inactivate endogenous proteases before adding the exogenous enzyme. Depending on the activity of enzyme, temperature, and other factors such as the target molecular weight range of resultant peptides, the reaction is allowed to proceed for a duration ranging from less than one hour up to several hours. The hydrolysis reaction is then terminated either by using a heat treatment or by adjusting pH, depending on the type of enzyme used. The supernatant after removing solids is often adjusted to neutral pH and then dehydrated to yield the powdered protein hydrolysates (Fig. 1.1).

1.4.1.1 Endogenous enzymes in Pacific hake fish muscle

The offshore stock of Pacific hake or Pacific whiting that inhabits the continental slope and shelf within the California current system from Baja California to British Columbia (BC), Canada (Quirollo 1992) is often infected by two species of *Kudoa*, a genus of myxosporean protozoan parasite, namely *Kudoa paniformis* and *Kudoa thyrsites* (Kabata and Whitaker 1985). Pacific hake muscle tissue infected with *K. paniformis* possess high cathepsin L-like protease activity (An and others 1994; Seymour and others 1994) which break down muscle and connective tissue proteins during post mortem and subsequent processing of hake fillets or mince. Cathepsin L is a lysosomal cysteine protease that prefers substrates with Arg and Lys at the P1 position and Phe, Trp, and Tyr at the P2 position (Choe

and others 2006). According to a study conducted by An and others (1994), the optimal conditions for Pacific hake protease activity were pH 5.50 and 55 °C, when tested using a crude fish fillet homogenate and the azocasein synthetic substrate. Further, purified cathepsin L from Pacific hake was capable of breaking down myofibrillar (especially myosin heavy chain) as well as stroma proteins extracted from Pacific hake fish fillet (An and others 1994). This endogenous protease could therefore be a good candidate for producing FPH through autolysis of Pacific hake fish fillet mince.

1.4.2 Optimizing process conditions

Careful choice of a suitable enzyme and hydrolysis conditions such as enzyme to substrate ratio, hydrolysis time, and temperature are crucial in obtaining protein hydrolysates with desirable functional and biological properties. Response Surface Methodology (RSM) experimental designs can be used in optimizing such process conditions as well as to predict properties of protein hydrolysates based on process conditions applied (Myers and Montgomery 2002; Minitab 2006). The RSM method has been applied to obtain the highest degree of hydrolysis (DH) of a dogfish muscle using Alcalase 2.4L® (Diniz and Martin 1996), to study the change of emulsification and foaming capacities of this hydrolysate as a function of DH over time (Diniz and Martin 1997), as well as to optimize the ACE-inhibitory activity of a whey protein hydrolysate (van der Ven and others 2002).

1.4.3 Concentrating, purifying, and identifying bioactive peptides from FPH

Crude protein hydrolysates may be further processed, for example by passage through ultrafiltration membranes, in order to obtain a more uniform product with the desired range of molecular mass (Pihlanto and Korhonen 2003). In large-scale production of hydrolysates, membrane technology may also be coupled with enzymatic hydrolysis in a continuous process, thereby reducing the cost by eliminating the need for heat or pH adjustment to inactivate the enzymes at the end of hydrolysis (Guérard 2007). Low molecular mass membrane cut-offs such as 500, 1000, and 3000 Da are useful for concentrating small peptides with different bioactivities from the higher molecular mass fractions and remaining

enzymes. Other techniques such as nanofiltration, ion-exchange membranes, or column chromatographic methods can be used in concentrating and purifying bioactive peptides based on the characteristics of the desired bioactive molecules (Pihlanto and Korhonen 2003).

Liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) followed by a database search (e.g. MASCOT method, Perkins and others 1999) is usually used in identifying peptide sequences. Matrix-assisted Laser Desorption/Ionization Time of Flight (MALDI-ToF) mass spectrometric analysis is also useful in obtaining peptide profiles of protein hydrolysates or semi-purified fractions. A major limitation of this method in obtaining peptide profiles of hydrolysates is that the peptides with molecular masses below 500 Da are difficult to identify since the α-cyano-4-hydroxycinnamic acid matrix molecules used for absorbing energy during analysis also appear in this region of the chromatogram (Garbis and others 2005). In addition, some peptides may not be ionized and "fly" with the matrix molecules and hence may not be detected. Amino acid analysis can also be conducted to quantify amino acid composition and purity of proteins or peptides, as well as the content of free amino acids present in a hydrolysate. Amino acid composition data are useful in identifying the presence of particular amino acids that are important for desired biological function of peptides, as well as in detecting atypical amino acids that might be present.

1.5 Antioxidative Peptides

A great deal of attention has appeared in recent literature to identify and assess antioxidative potential of peptides derived from various food sources. Even though there are few *in vivo* studies conducted to date, *in vitro* studies using various chemical assays have indicated the potential of these food- derived peptides to act as antioxidative agents to control various oxidative processes in food as well as in the human body.

1.5.1 Antioxidative peptides to improve human health

1.5.1.1 Endogenous antioxidative peptides

Under normal physiological conditions, human health is maintained by the balance of antioxidative and oxidative agents in the body. Reactive oxygen species (ROS) such as hydroxyl radicals ($^{\bullet}OH$), peroxyl radicals ($^{\bullet}OOR$), superoxide anion ($O_2^{-\bullet}$), and peroxynitrite ($ONOO^-$) are generated during normal biological processes (Decker and Xu 1998). Endogenous antioxidants help to protect tissues and organs from oxidative damage caused by these ROS. These endogenous antioxidative systems in the body include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, and various nonenzymatic compounds such as selenium, α-tocopherol, and vitamin C (Decker and Xu 1998). Apart from these, amino acids and peptides also contribute to the overall antioxidative capacity of cells and help in maintaining the health of biological tissues. Glutathione (γ-Glu-Cys-Gly), carnosine (β-alanyl-L-histidine), anserine (β-alanyl-L-1-methylhistidine), and ophidine (β-alanyl-L-3-methylhistidine) are antioxidative peptides naturally present in muscle tissues (Chan and Decker 1994; Babizhayev and others 1994). Glutathione acts as an electron donor to protect cells from free radicals (Bray and Taylor 1994). Carnosine on the other hand can act as a free radical scavenger as well as a metal ion chelator (Kim and others 2002). Carnosine demonstrated both *in vivo* and *in vitro* antioxidative activity in rat skeletal muscle lipid and protein components under the conditions of oxidative stress (Nagasawa and others 2001). Vasodilatory action of carnosine was also reported by Ririe and others (2000). Moreover, carnosine, anserine, and ophidine have been studied to determine potential physiological functions related to neurotransmitter synthesis (Snyder 1980).

1.5.1.2 Exogenous antioxidative peptides

The antioxidant-prooxidant balance in human body can change with the progression of age and due to other factors such as environmental pollutants, fatigue, excessive caloric intake, and high fat diets. With advancing age, the plasma and cellular antioxidant potential as well as the absorption of nutrients, including antioxidants, gradually diminishes (Rizvi and others 2006; Elmadfa and Meyer 2008) leading to an increased vulnerability of proteins to

free radical attack. Further, other environmental factors mentioned above could also weaken the body's immune system and make the body vulnerable to oxidative attack. Use of dietary antioxidants to promote human health by increasing the body's antioxidant load has been recognized as feasible and potentially effective.

The market of dietary antioxidant supplements has expanded exponentially over the past two decades. Most of these supplements contain antioxidants such as α-tocopherol, vitamin C, or plant-derived antioxidant compounds known as phytochemicals and plant extracts, e.g., lycopene, lutein, isoflavones, green tea extracts, and grape seed extracts. Use of antioxidant peptides and protein hydrolysates in these supplements is a relatively new trend and much of the research works in this area has been conducted within the last decade. Nevertheless, according to research findings so far, food products, supplements, or natural health products containing antioxidative peptides should be expected to command a huge market. However, few commercial products are available to date, which may be attributed to a variety of reasons including a lack of clinical trials confirming bioactivity, efficacy and safety, poor functional properties, high production cost, problems in making a reproducible product, bitterness or other organoleptic problems, and sometimes due to strict regulations and the lengthy procedures required to obtain approval for marketing the new product.

1.5.1.3 *In vivo* efficacy of antioxidative peptides

In order to exert the desired biological activity *in vivo*, peptides from food sources should reach target sites in the body without losing their activity. These peptides should therefore either be resistant to the GI enzyme degradation or should produce bioactive peptide fragments upon the GI digestion process. Enzymes such as pepsin, trypsin, α-chymotrypsin, elastase, and carboxypeptidase A and B are involved in breaking down of proteins during GI digestion (Vermeirssen and others 2004). The tetradecapeptide Leu-Val-Gly-Asp-Glu-Gln-Ala-Val-Pro-Ala-Val-Cys-Val-Pro released from *in vitro* GI digestion of mussel (*Mytilus coruscus*) protein exhibited potent antioxidative potential, inhibiting the formation of reactive oxygen species from the peroxidation of polyunsaturated fatty acids (Jung and others 2007). The ACE-inhibitory activity of two oligopeptides (Ile-Trp-His-His-Thr and Ile-Val-Gly-Arg-Pro-Arg-His-Gln-Gly) was shown to increase upon incubation with chymotrypsin and trypsin, respectively (Fujita and others 2000). However, breakdown into

smaller peptides and amino acids during GI digestion of bioactive peptides already present in food, prepared protein hydrolysates or isolated bioactive peptide fractions, can also occur, and may result in decrease of their bioactivities (Miguel and others 2006). Depending on the results of GI digestion and ACE or other enzyme action *in vivo*, bioactive peptides may be classified into pre-drug, pro-drug and substrate types of ACE inhibitors (Fujita and others 2000). For the "pre-drug" type ACE inhibitors, the antihypertensive activity *in vivo* must first be activated by hydrolysis with GI proteases and/or by ACE. ACE inhibitors in which the antihypertensive activity is increased by either GI digestion or the action of ACE are referred to as "pro-drug" type (Hasan and others 2006). Antihypertensive activity of "substrate" type ACE inhibitors is reduced by contact with ACE (Hasan and others 2006).

In order to be an effective bioactive compound, these peptides should either be able to pass through the intestinal wall in order to enter the blood stream and reach target organs, or modulate nutrient absorption and GI function through a direct effect in the gut lumen (e.g. caseinphosphopeptides) or through binding to specific epithelial cell surface receptor sites (e.g. opiod peptides) (Miguel and others 2008). Peptides such as the ones with ACE-inhibitory capacity should cross the intestinal epithelium in order to reach the peripheral organs (Vermeirssen and others 2002). To date, only a few studies have been conducted using *in vitro* Caco-2 cells in order to identify the transport mechanisms of antihypertensive peptides which already had been proven to show *in vivo* bioactivity by clinical trials or spontaneously antihypertensive rat assays (Shimizu and others 1997; Satake and others 2002; Vermeirssen and others 2002; Geerlings and others 2006; Miguel and others 2008; Quirós and others 2008). Small di- and tri-peptides may be absorbed intact across the brush border membrane using H^+-coupled PepT1 transporter system (Vermeirssen and others 2002). Large water soluble peptides can cross the intestinal barrier paracellularly via the tight junction between cells, while highly lipid-soluble peptides may diffuse via the transcellular route (Miguel and others 2008). Peptides may also enter the enterocytes via endocytosis, which entails membrane binding and vesiculisation of the material (Ziv and Bendayan 2000). The intestinal basolateral membrane also possesses a peptide transporter, which facilitates the exit of hydrolysis-resistant small peptides from the enterocyte into the portal circulation (Gardner 1984). Further, the contribution of each route and the ability of individual peptides to transport across the membrane, depend upon the molecular size, other structural

characteristics such as hydrophobicity, as well as their resistance to brush-border peptidases (Shimizu and others 1997; Satake and others 2002).

A limited number of research studies have been conducted to date in assessing antioxidative potential of peptide hydrolysates or isolated peptides using cell cultures, animal models or human clinical trials. Rajapakse and others (2005) reported that the radical scavenging peptide His-Phe-Gly-Asp-Pro-Phe-His derived from fermented mussel sauce could enhance the viability of oxidation induced cultured human lung fibroblasts cells by 76 % following 75 μg/mL of treatment. This effect was dose dependent up to 75 μg/mL, but showed no further protection on cell survival when the peptide concentration was increased beyond that. Seacure® is a commercially available fermented fish product made by controlled yeast fermentation of Pacific hake, which is claimed to be beneficial for a variety of gut conditions. Fitzgerald and others (2005) studied the efficacy of this product using various models of epithelial injury and repair. When cultured rat epithelia and human colon cells were given the Seacure®, cell growth was significantly increased (at 1 mg/ml concentration, $p< 0.01$) and the cell injury was significantly reduced (at 25 mg/ml concentration, $p< 0.05$) due to the action of ethanol soluble di- and tri-peptides containing glutamine (Fitzgerald and others 2005). A recent pilot human clinical trial using Seacure® pointed out that it could reduce the degree of small intestinal damage caused by the non-steroidal anti-inflammatory drug, indomethacin (Marchbank and others 2008). This study also suggested that glutamine present in FPH might have contributed to antioxidative activity via stimulation of glutathione production. In another *in vivo* study, the overall antioxidative status of hypertensive rats was improved by 35 % when they were fed with an FPH preparation compared to casein (Boukortt and others 2004). Sun and others (2004) reported that peptides isolated from medicinal mushroom *Ganoderma lucidum* showed antioxidant activity in the rat liver tissue homogenates and mitochondrial membrane peroxidation systems. The auto-hemolysis of rat red blood cells was also blocked by these peptides in a dose-dependent manner. In a human intervention study, ingestion of a *G. lucidum* supplement was found to cause an acute increase in plasma antioxidant capacity. However, no significant change in any biomarkers of antioxidant status in healthy human subjects was observed (Wachtel-Galor and others 2004).

1.5.2 Antioxidative peptides to control oxidative processes in food

Protein hydrolysates and peptide mixtures can also be used in food systems to reduce oxidative changes during storage. Incorporation of an FPH preparation made by autolysis of arrowtooth flounder protein into a coating of salmon fillets slowed down the lipid oxidation process (Sathivel 2005). Further, a brine solution containing salmon FPH injected into smoked salmon fish fillets was shown to reduce lipid oxidation measured as 2-thiobarbituric acid reactive substances (TBARS) during 6 weeks of cold storage (4 °C) and 8 months of frozen storage (-18 °C) (Hagen and Sandnes 2004). Protein hydrolysates made from whey, casein, egg yolk, and potato have also been shown to inhibit lipid oxidation in muscle foods (Pena-Ramos and Xiong 2003; Diaz and Decker 2005; Wang and Xiong 2005; Sakanaka and Tachibana 2006). Antioxidative caseinophosphopeptides, derived from tryptic digestion of casein, have been used in breakfast cereals, breads, pastry, chocolate, juices, tea and mayonnaise (FitzGerald 1998). In addition to inhibiting lipid peroxidation, peptides prepared by enzymatic protein hydrolysis are capable of preventing oxidative modification of intact proteins. For example, the presence of potato protein hydrolysate minimized amino acid side chain damage and structural changes in myofibrillar proteins exposed to a $^{\bullet}OH$-generating oxidizing system (Wang and Xiong 2008).

1.5.3 Mechanism of antioxidative action of proteins, peptides, and amino acids

In general, the term "antioxidant" can be defined as "any substance that when present at low concentrations, compared with those of oxidizable substrate, significantly delays or inhibits oxidation of that substrate" (Gutteridge 1994). Dietary antioxidants can scavenge ROS and reactive nitrogen species (RNS) to stop radical chain reactions (sacrificial), or can inhibit the formation of reactive oxidants (preventive) (Huang and others 2005). The formation of free radicals may be inhibited by reducing hydroperoxides and chelating metal ions (Niki 2002). Radical scavenging action on the other hand depends upon the reactivity and concentration of antioxidant as well as the stability of the resultant antioxidative radical (Apak and others 2007).

In general, all 20 amino acids found in proteins can interact with free radicals if the energy of the free radical is high (e.g. hydroxyl radicals) (Elias and others 2008). In particular, amino acids such as Cys and Met that contain nucleophilic sulfur-containing side chains, and Trp, Tyr, and Phe that have aromatic side chains can easily donate hydrogen atoms. The imidazole-containing side chain in His also is oxidatively labile. However, free amino acids are not generally found to be effective as antioxidants in food and biologically systems and extensive proteolysis of food proteins in fact has resulted in decreased antioxidative activity (Chan and others 1994; Ostdal and others 1999; Zhou and Decker 1999; Rival and others 2001). The increased antioxidative activity of peptides compared to free amino acids is related to the unique properties contributed by their chemical composition and physical properties, especially the stability of the resultant peptide radicals that do not initiate or propagate further oxidative reactions (Elias and others 2008). Nevertheless, a recent study conducted by Tsopmo and others (2009) claims that Trp release from human milk may have potential to act as a powerful radical scavenger.

Antioxidative mechanism of peptides has been postulated to be based on their metal ion chelation activity, free radical scavenging capacity, and aldehyde adduction activity (Chen and others 1996; Zhou and Decker 1999). The majority of the antioxidative peptides derived from food sources have molecular weights ranging from 500 to 1800 Da (Wu and others 2003; Jun and others 2004; Je and others 2005a,b; Ranathunga and others 2006, Kim and others 2007) and include hydrophobic amino acid residues of Val or Leu at the N-terminus of the peptides, and Pro, His, Tyr, Trp, Met, and Cys in their sequences (Uchida and Kawakishi 1992; Chen and others 1995; Elias and others 2006).

Hydrophobic amino acid residues like Val or Leu can increase the interaction between the peptide and fatty acids in order to scavenge free radicals generated at the lipid phase (Ranathunga and others 2006). Although the structure-activity relationship of antioxidative His-containing peptides has not been well defined yet, the activity could be attributed to hydrogen donating ability, lipid peroxy radical trapping, and/or the metal ion-chelating ability of the imidazole group (Chan and Decker 1994). The differences in the activity of individual His-containing peptides may be due to the environment surrounding the imidazole group, as indicated by various observations. Murase and others (1993) found that *N*-(longchain-acyl) histidine-containing compounds suppressed the oxidation of

phosphatidylcholine liposomes and methyl linoleate. The hydrophobicity of the compounds was important for the accessibility to the hydrophobic targets.

Uchida and Kawakishi (1992) investigated the oxidation of Asp-Arg-Val-Tyr-Ile-His-Pro-Phe mediated by copper (II)/ascorbate. The N-terminal Asp-Arg-Val-Tyr sequence contributed significantly to the reactivity of the His residue, which was converted to the 2-imidazolone derivative upon oxidation. Without the N-terminal segment, His residue in Ile-His-Pro-Phe showed no reactivity against the oxidation. Chen and others (1996) measured antioxidative activities of 28 synthetic peptides designed based on an antioxidative peptide (Leu-Leu-Pro-His-His) derived from a proteolytic digest of soybean protein. Results indicated that removal of C-terminal His residue decreased antioxidative activity, whereas removal of N-terminal Leu had no effect. In the peptide sequence, His and Pro played important roles in the antioxidative activity, and among the peptides tested, Pro-His-His was the most antioxidative. Further, peptides with the Pro-His-His sequence had the greatest synergism with lipid-soluble antioxidants, e.g., tocopherols and BHA. Saito and others (2003) also studied antioxidative activity of peptides created in two tripeptide libraries. According to their results, out of the 108 peptides containing either His or Tyr residues, two Tyr-containing tripeptides showed higher activity than those of two His-containing tripeptides in the linoleic acid peroxidation system. Further, Tyr-His-Tyr showed strong synergistic effects with phenolic antioxidants. Similar to results reported by Chen and others (1996), out of the 114 synthesized tripeptides containing Pro or His, Pro-His-His exhibited the greatest antioxidative activity. Moreover, substitution of other amino acid residues for either the N-terminus or C-terminus of the tripetide did not significantly alter its antioxidative activity. Cys-containing tripeptides on the other hand showed a strong peroxynitrite scavenging activity. Tripeptides containing Trp or Tyr residues at the C-terminus had strong radical scavenging activities, but weak peroxynitrite scavenging activity. In another study, Tsuge and others (1991) reported the isolation of a potent antioxidative peptide Ala-His-Lys from the egg white albumin hydrolysate, in which neither His-Lys nor a constituent amino acid mixture had any activity, but Ala-His was as potent as the parent tripeptide. These results demonstrate the importance of amino acid composition, sequence (Chen and others 1998), and size (Peña-Ramos and others 2004) in determining the antioxidative potential of peptides.

In addition to peptides derived from food proteins, proteins themselves have the ability to act as antioxidative agents by inactivating reactive oxygen species, scavenging free radicals, chelating prooxidative transition metals, reducing hydroperoxides, and enzymatically eliminating specific oxidants (Elias and others 2008). Proteins such as casein, β-lactoglobulin, and lactoferrin have been found to be antioxidative in various food systems (Diaz and Decker 2005; Elias and others 2005; Kong and Xiong 2006). In food emulsion systems, proteins and peptides in protein hydrolysates can reach to the oil-water interface due to their surface active property and can form a membrane or coating to prevent contact of lipids with oxidizing agents. This physical barrier can also help in reducing lipid peroxidation in food systems. Donnelly and others (1998) reported that the formation of a thick protein membrane by whey proteins used as the emulsifier instead of Tween 20 helped in improving oxidative stability of emulsified Menhaden oil. Acidic conditions are also preferred in emulsions made with proteins due to the fact that protonated amino groups can repel cationic prooxidants such as Fe^{2+} and Cu^{2+} and thereby inhibit the initiation of lipid peroxidation (Kellerby and others 2006). However, due to their large size, proteins can not pass through cellular membranes in order to act as biologically active compounds unless they generate smaller antioxidative peptides upon degradation by GI enzymes or brush border peptidases.

1.5.4 Measuring the antioxidative potential of peptides

Specific assays have not yet been developed or standardized to measure antioxidative capacity of peptides or peptide mixtures. Therefore, assays that are commonly used in measuring antioxidative capacity of non-peptidic antioxidants have been used in the literature to measure the antioxidative capacity of peptides as well. *In vitro* assays based on chemical reactions are widely used in quantifying antioxidative effectiveness of whole food, partially purified peptides, and/or individual peptides isolated from food mixtures in preventing oxidative processes occurring in human body as well as in food systems during storage. Even though these chemical assays give an insight to the potential biological activity of these food-derived antioxidants, further analysis such as investigating the fate of peptides during gastrointestinal (GI) digestion, their ability to penetrate through cellular membranes, as well

as their *in vivo* stability and reactivity has to be conducted in order to confirm their biological activity. On the other hand, when considering applications of antioxidative compounds to control oxidative rancidity in complex food systems, the physical location (e.g. does the antioxidative compound concentrate where the oxidative reactions are most prevalent) is also an important parameter to be considered (Alamed and others 2009).

1.5.4.1 *In vitro* chemical assays

Even though it is important to identify and quantify individual antioxidants present in a desired food mixture, separating antioxidative compounds from the complex food system and studying each one individually is costly and inefficient. Further, possible synergistic interactions among antioxidants in food mixture will be compromised during this procedure. Therefore, it is very appealing to have a convenient assay method for the quick quantification of antioxidative effectiveness of whole food or partially purified antioxidant fractions. However, due to the complexity of different oxidative processes occurring in food or biological systems as well as the possibility of having different antioxidative mechanisms by various antioxidative compounds, finding one method that can show the overall antioxidative potential of food is not an easy task. Nevertheless, methods such as the Trolox equivalent antioxidant capacity (TEAC) assay, oxygen radical absorbance capacity (ORAC) assay, and the total radical-trapping antioxidant parameter (TRAP) assay have been widely reported in the literature for measuring antioxidative capacity of food and biological samples (Cao and Prior 1998; Re and others 1999; Ghiselli and others 2000). These methods plus other commonly used antioxidative capacity assays are listed in Table 1.1. Based on the chemical reactions involved, hydrogen atom transfer reaction (HAT) based assays quantify hydrogen atom donating ability of an antioxidant whereas the electron transfer (ET) based assays measure the reducing capacity.

The ORAC assay is an example of a HAT-based assay (Table 1.1) that applies a competitive reaction scheme where antioxidant and substrate (fluorescein probe) kinetically compete for artificially generated hydroxyl radicals (Jimenez-Alvarez and others 2008). As the reaction progresses, the decrease of fluorescence due to consumption of fluorescein is retarded by the antioxidant compound present. Antioxidative capacity is calculated by the difference in the area under the curve (AUC) for blank and the sample (Jimenez-Alvarez and

others 2008). The ORAC-fluorescein assay is conducted in an aqueous system and does not give an estimation of the antioxidative activity of lipophilic antioxidants (Huang and others 2002). Therefore a modification has been made to this assay using randomly methylated β-cyclodextrin (RMCD) as the molecular host to enhance the solubility of lipophilic antioxidants (Huang and others 2002).

Inhibition of linoleic acid autoxidation (Table 1.1) by antioxidants also takes place via HAT reactions. The main steps involved in lipid autoxidation that is initiated by an azo compound, and the action of antioxidants in inhibiting and terminating the radical generation process, are shown in Figure 1.2. During the assay, the extent of lipid peroxidation with and without the presence of antioxidant can be measured by using the ferric thiocyanate assay (Kikuzaki and Nakatani 1993). Lipid hydroperoxides in the reaction media can convert Fe^{2+} of the ferrous thiocyanate to Fe^{3+} in order to produce the red colored ferric-thiocyanate complex that can be measured spectrophotometrically at 500 nm. Secondary lipid oxidation products such as hexanal, malonaldehyde, and other carbonyl compounds formed by break down of lipid hydroperoxides can also be measured in assessing the extent of lipid oxidation in the linoleic acid autoxidation system. Gas chromatographic (GC)-headspace analysis can be used in quantifying the amount of hexanal formed during storage (Snyder and others 1985) while thiobarbituric acid (TBA) assay can be used to quantify TBA-reactive substances (TBARS) (Gray and Monahan 1992).

The TEAC assay is based on an ET reaction (Table 1.1). During the reaction, the blue/green ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation, that has absorption maxima at 645 nm, 734 nm and 815 nm, is generated through a reaction between ABTS and potassium persulfate. In the presence of an antioxidant, pre-formed radical cation is reduced to ABTS changing color of the reaction solution from blue to colorless. The extent of decoloration at 734 nm (the preferred wavelength due to less interference from pigments) after 6 min as a function of antioxidant concentration is measured and the TEAC value is obtained by calculating the concentration of antioxidant giving the same percentage inhibition of absorbance of the radical cation as 1 mM of Trolox (Re and others 1999). This assay can be used in measuring antioxidative capacity of both water-soluble and lipid-soluble pure compounds as well as food extracts (Re and others

1999). TEAC is however an end-point assay and therefore the reaction rate differences between antioxidants and oxidants are not reflected (Huang and others 2005).

The FRAP assay is also based on ET-reaction (Table 1.1) and measures the ability of an antioxidant to reduce a ferric 2,4,6-tripyridyl-*s*-triazine salt (Fe^{3+}-TPTZ) to the blue colored ferrous complex (Fe^{2+}-TPTZ) at low pH. Even though the TEAC assay and FRAP assay provide similar results due to similar redox potentials of Fe^{3+}-TPTZ salt and ABTS radical cation (0.70 and 0.68 V, respectively) (Huang and others 2005), the FRAP assay pH is much lower (pH 3.6) than in the TEAC assay (pH 7.4).

Apart from these most commonly used assays, there are other chemical assays that measure the scavenging capacity of individual ROS such as superoxide anion, singlet oxygen, hydrogen peroxide, hydroxyl radical, and peroxynitrite (Huang and others 2005). Electron spin resonance (ESR) spectrometry is another method that has emerged as a useful tool in studying radical-antioxidant interactions and estimating free radical scavenging capacities of antioxidants (Je and others 2007). This technique involves an interaction between an applied magnetic field and chemical species with unpaired electrons such as free radicals and transition metal ions (Yu and Cheng 2008). This assay is specific with a clear reaction mechanism, is simple, and free of interferences from sample matrix components such as pigments (Yu and Cheng 2008). Direct ESR measurement can be performed using a stable radical species such as 2,2-diphenyl-1-picrylhydrazyl (DPPH·) radical, whereas ESR spin-trapping method with an exogenous spin-trapping molecule is used to measure short-lived radicals such as ROS (Yu and Cheng 2008).

Depending on the nature of the dietary antioxidant, it can also act as a metal ion chelator. Iron chelating activity can be measured using a spectrophotometric method where free Fe^{2+} ions present after addition of the antioxidant to the reaction mixture can be quantified by formation of a colored complex with ferrozine (Decker and Welch 1990).

Since the mechanism of antioxidative action measured and/or reaction conditions used are different from one assay to another, an antioxidative compound can give different results depending on the assay system used. For example, the reducing capacity of an antioxidant can be affected by pH of the assay media. The assays of FRAP, TEAC, and Folin-Ciocalteu reagent (FCR) (Table 1.1) that measure the reducing capacity of antioxidants use acidic, neutral, and basic conditions, respectively (Huang and others 2005). Further, a good ion

chelator may not show any activity in radical scavenging assays mentioned above. Solubility of antioxidants in the reaction media is also playing an important role in determining antioxidative capacity. Due to these reasons, more than one assay is often used in measuring antioxidative capacity of a food or a food constituent of interest.

1.5.4.2 Cell culture systems

In vitro cultured cell model systems allow for rapid, inexpensive screening of antioxidative compounds for their bioavailability and metabolism, compared to expensive and time-consuming animal studies and human clinical trials. Human adenocarcinoma colon cancer (Caco-2) cell monolayers have been the most commonly reported in the literature for studying intestinal permeability of bioactive compounds due to their similarity to the intestinal endothelium cells (Vermeirssen and others 2005; Liu and Finley 2005). Upon culturing as a monolayer, Caco-2 cells differentiate to form tight junctions between cells to serve as a model of paracellular movement of compounds across the monolayer. Further, Caco-2 cells express transporter proteins, efflux proteins, and Phase II conjugation enzymes to model a variety of transcellular pathways as well as metabolic transformation of test substances (van Breemen and Li 2005). Semipermeable plastic supports that can be fitted into the wells of multi-well culture plates are usually used in culturing Caco-2 cells. Test compounds are then added to either the apical or basolateral sides of the monolayer. After incubation for various lengths of time, aliquots of the buffer in opposite chambers are removed for the determination of the concentration of test compounds and the computation of the percentage permeability for each compound through RP-HPLC analysis.

Cell culture models can also be used to evaluate cytotoxicity of antioxidative compounds at concentrations to be used to exert the desired bioactivity in the body, as well as to study the potential to inhibit intracellular oxidation and to reduce inflammatory responses. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which measures the metabolic activity of cells through oxidation-reduction activities of mitochondria, is often used in measuring the viability of cells during cytotoxicity assays (Mosmann 1983). During experiments, intracellular oxidation of cells can be induced by using the AAPH peroxy radical generator or by using hydrogen peroxide (Elisia and Kitts 2008). The 2′,7′-dichlorofluorescein diacetate (DCFH-DA) probe is used in measuring the

extent of radical formation with and without added antioxidative compounds. Upon cellular uptake, the DCFH-DA probe is hydrolyzed to DCFH by intracellular esterases. DCFH is capable of emitting fluorescence when oxidized by peroxy radicals during the assay. The extent of oxidation can therefore be measured by the fluorescence intensity with and without the presence of antioxidative compounds (Hu and others 2005).

1.5.4.3 *In vivo* assays

Once the antioxidative potential of a food constituent of interest is established using *in vitro* assay methods described above, animal studies and human clinical trials can be conducted to confirm the desired biological function. Results from these *in vivo* assays are an essential part in gaining approval from federal agencies for a dietary component to be used in functional food and nutraceutical formulations (Health Canada 2003). However, as described in Section 1.5.1.3, few animal studies and clinical trials on antioxidative protein hydrolysates and peptides have been conducted so far. While promising results have been observed for many of the clinical trials conducted using non-peptidic low molecular weight antioxidants to assess their ability to prevent cardiovascular diseases, different types of cancer, degenerative disorders, and overall mortality, other studies have failed to show any long term beneficial effect (Lotito and Frei 2004; Etminan and others 2005; Pham and Plakogiannis 2005). Therefore, the use of food derived exogenous antioxidants in general to improve human health needs to be assessed further for their effectiveness and mechanisms involved.

1.6 Antioxidative Peptides from Fish

1.6.1 Naturally occurring antioxidative peptides from fish

Carnosine (β-alanyl-L-histidine), anserine (β-alanyl-L-1-methylhistidine), and ophidine (β-alanyl-L-3-methylhistidine) are three antioxidative dipeptides naturally present in skeletal muscle tissues (Chan and Decker 1994). However, the amounts of these peptides present can vary greatly in different animals. For example, the contents of anserine present in chum

salmon, Siberian salmon, and trout were 1273, 1020, and 51.6 mg/100 g tissue, respectively (Boldyrev 1987; Boldyrev and Severin 1990). None of these species had significant amounts of carnosine and ophidine in their muscle tissues. Blue whale and dolphin muscle tissues contained only carnosine and ophidine, whereas other marine animals such as crab, oyster, and squid did not have any of these endogenous dipeptide antioxidants in the muscle (Boldyrev 1987; Boldyrev and Severin 1990). Amounts of these naturally occurring antioxidants are also found to depend on the age of the animal and the diet (Chan and Decker 1994).

Several studies have demonstrated that the cytosolic aqueous fraction of fish muscle can inhibit *in vitro* oxidation of isolated membrane systems such as flounder sarcoplasmic reticulum (Erickson and others 1990), herring microsomes (Slabyj and Hultin 1983), and trout microsomes (Han and Liston 1987). Undeland and others (2003) further studied this phenomenon using an aqueous fraction (press juice) of cod muscle and indicated that this fraction could inhibit hemoglobin-mediated oxidation of the lipids of washed cod mince during ice storage. Undeland and others (2003) suggested that low molecular weight non-enzymic compounds are involved in targeting free radicals, membrane surfaces, or hemoglobin, but the specific compounds responsible for these antioxidative activities are yet to be identified.

1.6.2 Antioxidative peptides derived from fish

Table 1.2 is a compilation of recent research published on antioxidative properties of fish-derived protein hydrolysates; this is a very active area of research with new findings continuing to be reported in the literature. Some of these studies further fractionated the hydrolysates in order to isolate and identify individual antioxidative peptides (Table 1.2). For example, Jao and Ko (2002) identified seven antioxidative peptides from a tuna cooking juice hydrolysate (Table 1.2) with potential radical scavenging abilities towards DPPH. A peptide with strong radical scavenging activity was identified from a tryptic hydrolysate of hoki skin gelatin (Table 1.2) which possessed antioxidative activity against linoleic acid peroxidation close to that of the synthetic antioxidant butylated hydroxytoluene (BHT). This peptide could also increase the antioxidative enzyme levels in cultured human hepatoma cells due to the involvement in maintaining the redox balance in the cell environment (Mendis and others

2005). Potential toxicity of the fish-derived peptides for human use was tested using human lung fibroblast and human endothelial cell lines and an isolated peptide from a peptic hydrolysate of tuna backbone protein by Je and others (2007) (Table 1.2); no cytotoxic effect was found at any of the concentrations tested (1 to 1000 μg/ml).

1.7 Multi-functional Peptides

Recent research revealed that some protein hydrolysates contain peptides possessing several different biological activities. For example, protein hydrolysates made from fermented milk (Hernández-Ledesma and others 2005), human milk and infant formula (Hernandez-Ledesma and others 2007), chum salmon cartilage (Nagai and Nagashima 2006), and egg albumen (Davalos and others 2004) contained both antioxidative as well as ACE-inhibitory peptides. Further, peptides from the sequence 60-70 of the β-casein have shown immunostimulatory, opioid, and ACE-inhibitory activities (Korhonen and Pihlanto 2003). These protein hydrolysates with different bioactivities may be therefore used as multifunctional ingredients in functional foods to control various human diseases.

The ACE-inhibitory activity is the most studied biological activity for the peptides derived from fish. Studies conducted with animal models and, more importantly, in human clinical trials conducted with mildly hypertensive subjects, have demonstrated a great potential in reduction of blood pressure following daily ingestion of fish-derived ACE inhibitors (Meisel and others 2006). For example, due to its promising antihypertensive effect, the oligopeptide Leu-Lys-Pro-Asn-Met derived from a thermolysin digest of dried bonito (Fujita and Yoshikawa 1999) has been officially approved for use under the "Food for Specified Health Use" category in Japan (Vercruysse and others 2005), and was filed with the U.S. Food and Drug Administration (FDA) as a new dietary supplement ingredient (U.S. FDA 2001).

1.8 Research Hypotheses

Given an overall objective of investigating potential utilization of Pacific hake as a source of antioxidative peptides, the hypotheses of the present research are as follows:

1. The extent of *Kudoa paniformis* infection can be correlated with proteolytic degradation and textural changes in Pacific hake fish muscle.
2. Endogenous proteolytic activity of parasitized Pacific hake fish fillet mince can be used in production of FPH with antioxidative properties which will be at least as effective as FPH made using the commercial enzyme preparations of Validase® and Flavourzyme®.
3. Other than antioxidative capacity, FPH made from Pacific hake will also possess ACE-inhibitory activity.
4. *In vitro* simulated GI digestion and cell culture permeability studies will demonstrate potential bioavailability of the antioxidative and ACE-inhibitory capacity of peptides in FPH.
5. Sequence and molecular characteristics of peptides will affect their antioxidative capacity in different (i.e. polar, non-polar, and emulsion) model systems.

1.9 Specific Objectives and Research Plan

The objectives and research plan of studies conducted to test the five research hypotheses mentioned in Section 1.8 are listed below.

Study 1. Correlation of *Kudoa* infection level with endogenous proteolytic activity and texture of fish mince from Pacific hake

- Optimize the temperature and pH conditions for proteolytic activity of endogenous proteases present in Pacific hake fillet mince.

- Study the influence of *Kudoa* parasitization on endogenous proteolytic activity and protein degradation in Pacific hake.
- Investigate the potential of using *Kudoa* spore counts as an indicator of infection level, proteolytic degradation, and texture in Pacific hake fish mince.

Study 2. Production of FPH from Pacific hake fish mince and characterization of antioxidative peptides

- Produce a set of FPH through autolysis using Pacific hake fish fillet mince and study antioxidative properties of FPH using different *in vitro* assay systems.
- Compare FPH produced through autolysis to FPH produced with the addition of commercial enzymes.
- Identify one or several FPH with promising antioxidative activity based on antioxidative activity assay results.
- Fractionate FPH using ultrafiltration and different chromatographic methods and identify peptide fractions and/or individual peptides responsible for antioxidative activity using LC-MS/MS.
- Compare antioxidative activity of FPH and isolated peptides to the activity of known antioxidants.

Study 3. Assessment of antioxidative FPH and ultrafiltrated fractions to act as ACE-inhibitors.

- Measure the *in-vitro* ACE-inhibitory activity of FPH and ultrafiltered fractions with antioxidative activity, which were produced in Study 2.

Study 4. *In vitro* study of bioavailability and bioactivity of the antioxidant and ACE-inhibitory peptides in FPH

- Study the effect of simulated GI-digestion on antioxidative and ACE-inhibitory capacity of FPH and semi-purified peptide fractions.

- Study the ability of antioxidative and ACE-inhibitory peptides from FPH (before and after *in vitro* GI-digestion) to pass through Caco-2 cell monolayer and measure *in vitro* bioactivities of cell permeates.

Study 5. Antioxidative activity of purified antioxidative peptides in different model systems and their structure-activity relationships

- Synthesize antioxidative peptide sequences identified in Study 2 and selected antioxidative peptide sequences from the reported literature.
- Study the antioxidative capacity of synthetic peptides using different *in vitro* chemical assays.
- Study the effect of sequence and molecular characteristics of peptides on antioxidative potential in different *in vitro* assay systems.

Table 1.1 *In vitro* antioxidative capacity assays

Assays involving hydrogen atom transfer reactions[1]	ORAC (oxygen radical absorbance capacity)
$ROO\cdot + AH \longrightarrow ROOH + A\cdot$	TRAP (total radical trapping antioxidant parameter)
$ROO\cdot + LH \longrightarrow ROOH + L\cdot$	Crocin bleaching assay
	IOU (inhibited oxygen uptake)
	Inhibition of linoleic acid oxidation
	Inhibition of LDL oxidation
Assays by electron-transfer reaction[1]	TEAC (Trolox equivalent antioxidant capacity)
$M(n) + e \text{ (from AH)} \longrightarrow AH^{+\cdot} + M(n-1)$	FRAP (ferric ion reducing antioxidant parameter)
	DPPH (diphenyl-1-picrylhydrazyl)
	Copper (II) reduction capacity
	Total phenols assay by Folin-Ciocalteu reagent (FCR)
Other assays	TOSC (total oxidant scavenging capacity)[2]
	Inhibition of Briggs-Rauscher oscillation reaction[3]
	Chemiluminescence[4]
	Electrochemiluminescence[5]

AH = Antioxidant, LH = Substrate

[1]Huang and others (2005)

[2]Winston and others (1998)

[3]Cervellati and others (2002)

[4]Ashida and others (1991)

[5]Chmura and Slawinski (1994)

Table 1.2 Some antioxidative protein hydrolysates and peptides derived from fish

Source	Method of preparation[1]	Peptide sequence	Reference
Dried bonito	Molsin F	VKL, VVKLVKV, PKAVIKL, VPSGKEAK, FVAGKKAI, KVI, KD	Suetsuna (1999)
Sardine muscle	Pepsin	LQPGQGQQ	Suetsuna and Ukeda (1999)
Alaska pollack skin gelatin	Alcalase, and then 10 kDa molecular weight cutoff membrane permeate was hydrolyzed with Pronase E	GEOGPOGPOGPOGPOG[2] GPOGPOGPOGPOG[2]	Kim and others (2001)
Allaska pollack frame protein	Mackerel intestine crude enzyme	LPHSGY	Je and others (2005c)
Yellowfin sole frame protein	Mackerel intestine crude enzyme + pepsin	RPDFDLEPPY	Jun and others (2004)
Tuna cooking juice	Protease XXIII	PSHDAHPE SHDAHPE VDHDHPE PKAVHE PAGY PHHADS VDYP	Jao and Ko (2002)
Tuna back bone	Pepsin	VKAGPAWYANQQLS	Je and others (2007)
Mackerel fillet	Autolysis or Protease N	-[3]	Wu and others (2003)
Chum salmon cartilage and skin	Extract prepared using a pressure cooker	-	Nagai and Nagashima (2006)

Source	Method of preparation[1]	Peptide sequence	Reference
Salmon flesh remnants on frames after filleting	Protamex™	-	Berge (2005)
Capelin whole fish	Alcalase®	-	Amarowicz and Shahidi (1997)
Hoki skin gelatin hydrolysate	Trypsin	HGPLGPL	Mendis and others (2005)
Hoki frame protein	Pepsin	-	Je and others (2005a)
Hoki frame protein	Pepsin	ESTVPERTHPACPDPN	Kim and others (2007)
Conger eel muscle	Trypsin	LGLNGDDVN	Ranathunga and others (2006)
Round scad muscle(a)	Alcalase® 2.4L or Flavourzyme® 500L	-	Thiansilakul and others (2007a)
Round scad muscle (b)	Flavourzyme®	-	Thiansilakul and others (2007b)
Yellow stripe trevally hydrolysate	Alcalase® 2.4L or Flavourzyme® 500L	-	Klompong and others (2007)
Tilapia protein	Amano A2, Amano N, Flavourzyme®, Neutrase®, or Cryotin-F	-	Raghavan and others (2008)
Channel catfish protein isolates	Protamex™	-	Theodore and others (2008)

[1]In studies where hydrolysis was done by adding exogenous enzymes, the enzyme(s) used are mentioned

[2] "O" in these sequences indicate hydroxyproline

[3]Peptide sequences not identified

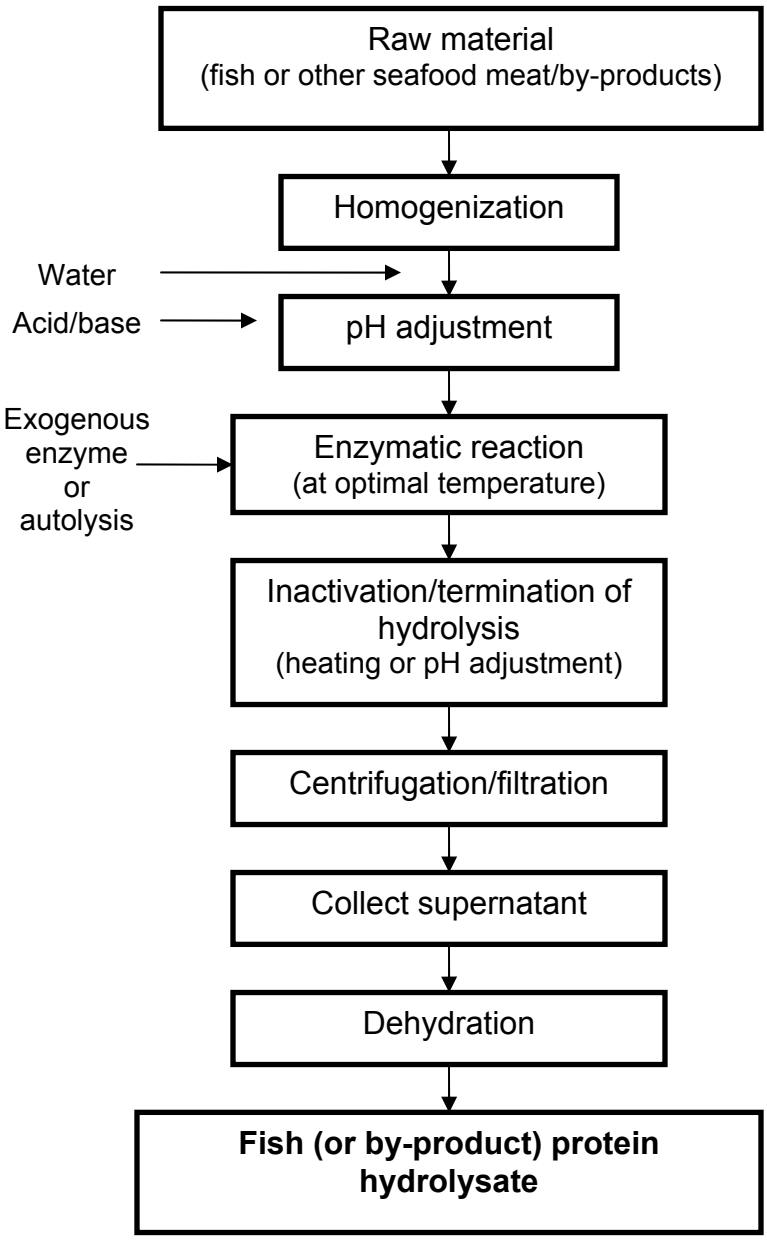

Figure 1.1 Flow diagram for the preparation of protein hydrolysates from fish and fish by-products

Adapted and modified from Kristinsson and Rasco 2000; Guérard and others 2005.

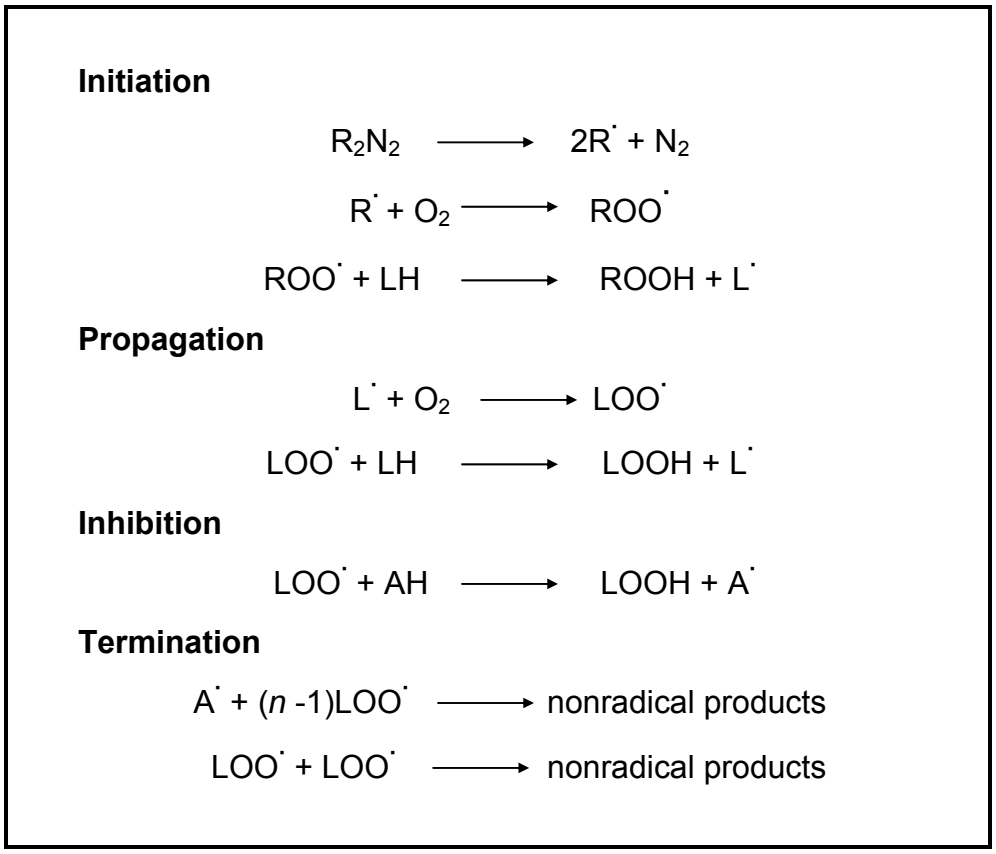

Figure 1.2 Autoxidation process initiated by an azo compound and the action of antioxidants. R_2N_2 = azo compound, LH = lipid substrate, AH = antioxidant Adapted from Denisov and Khudyakov 1987.

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CHAPTER 2 Correlation of *Kudoa* Infection Level with Endogenous Proteolytic Activity and Texture of Fish Mince from Pacific Hake[1]

2.1 Introduction

Pacific hake, *Merluccius productus*, is a member of the cod family with potential economic importance. The white flesh and favorable taste of Pacific hake are advantageous for many applications in the seafood processing industry where other white fish species such as pollock and cod are traditionally used. Furthermore, the high proportion of polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA), present in muscle tissue lipids of *M. productus* compared to other hake species, such as *M. hubbsi*, *M. australis*, *M. bilinearis*, *M. paradoxus* and *M. capensis,* or compared to Pacific and Atlantic cod (Wessels and Spark 1973; Kinsella 1987; Vlieg and Body 1988; Menedez and Gonzalez 1997) makes it a healthy alternative to these white fish that are often consumed in high quantities worldwide.

However, the offshore stock of Pacific hake that inhabits the continental slope and shelf within the California current system from Baja California to British Columbia (BC), Canada (Quirollo 1992) is often infected by two species of *Kudoa*, a genus of myxosporean protozoan parasite, namely *Kudoa paniformis* and *Kudoa thyrsites* (Kabata and Whitaker 1985). Pacific hake muscle tissue infected with *K. paniformis* possess high cathepsin L-like protease activity (An and others 1994; Seymour and others 1994) which break down muscle and connective tissue proteins during the post mortem period and subsequent processing of hake fillets or mince. This phenomenon, referred as "myoliquefaction", together with the visible black and/or white streaks present in infected fish flesh greatly affects the marketability of Pacific hake (Kabata and Whitaker 1985).

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In 2003, Pacific hake constituted BC's largest single fish species harvest by weight at 69,100 tonnes, which accounted for 31 per cent of the provincial total commercial harvest and 55 per cent of the ground fish harvest (The British Columbia Seafood Industry Year in Review 2003). Despite the precautions taken to reduce post mortem quality loss of Pacific hake, the potential formation of soft texture is an ongoing problem that affects the price and market value of this fish worldwide. Furthermore, the demand for hake surimi in both the U.S.A and Canada has decreased in recent years. This is related to concerns over bovine spongiform encephalopathy and the use of beef plasma protein (BPP) as the protease inhibitor in hake surimi (Porter 1992). Thus, in conjunction with alternative methods of utilization of infected fish, a method that can rapidly identify *Kudoa* infection level and that can be related to possible quality loss of Pacific hake is necessary in order to increase the market value of this underutilized fish. Moreover, the majority of literature data on *Kudoa* infection level and migration patterns of Pacific hake caught in Canadian waters was reported twenty or thirty years ago. Thus, new studies are necessary to obtain recent information on types and extents of *Kudoa* parasite infection of Pacific hake caught near BC, Canada, and their possible influence on quality.

Experimental evidence in the literature indicates that the visual culling method may have some potential use in sorting Pacific hake according to the level of infection (Patashnik and others 1982; Kudo and others 1987; Morrissey and others 1995). Visual white pseudocyst counts of Pacific hake muscle tissue have shown a positive correlation with protease activity and texture quality (Kudo and others 1987; Morrissey and others 1995). However, detection of the white pseudocysts requires careful examination. Thus, evaluation of even a few fish as a random sample would be too time-consuming and impractical for use on a production line. Also, infected tissues with black pseudocysts have shown higher proteolytic activity even though the correlation of black pseudocysts with proteolytic activity was low (Morrissey and others 1995). Another important factor is that pseudocysts are only one of the few stages in the *Kudoa* life cycle that can trigger proteolytic activity within Pacific hake muscle tissues (Nelson and others 1985). Therefore, this visual culling method may not provide an accurate indication of the level of proteolytic activity of infected Pacific hake fish, even though it can be used as an effective tool in removing heavily infected fillets with black streaks from the production line.

St-Hilaire and others (1997) introduced a spore counting method to be used for the prediction of parasite burdens that affect fillet quality in salmon populations. The test was found to be a good indicator for heavily infected fish. However, it was less satisfactory for lighter infections (St-Hilaire and others 1997). Dawson-Coates and others (2003) improved this method by taking spore counts from multiple fillet sites, and by using trypsin digestion of the muscle tissue in order to release *Kudoa* spores for easier enumeration. According to Dawson-Coates and others (2003), a mean *Kudoa* spore count, estimated by taking multiple samples from several locations of the fish, gave the best prediction of farmed Atlantic salmon fillet degradation caused by *K. thyrsites*. Further, a mean spore count of 4.0×10^5 per gram of tissue was found to be the spore level at which the probability of severe myoliquefaction of infected salmon becomes a significant risk (Dawson-Coates and others 2003). However, this approach of calculating a mean spore count based on multiple tissue samples from each fish would not be practical in a processing plant where thousands of fish could be filleted and minced each day, since random sampling of even 1 % would necessitate the testing of tens of fish, each in 4 to 8 tissue sites. It has been reported that tissue samples taken from upper napes are the most representative area of *Kudoa* infection (Morrissey and others 1995), but it is not known whether or not spore counts based on the nape area would be indicative of spore counts in the minced fillet.

The objectives of the present work were to study possible correlations of *Kudoa* spore counts, proteolytic activity, and cooked textural quality of fish mince prepared using fillets from Pacific hake caught in Canadian waters of the BC coast. Applicability of the improved spore counting method developed by Dawson-Coates and others (2003) to evaluate *Kudoa* spore counts in fish fillet mince as an indicator for the average level of parasite infection of the individual fish used to prepare particular batches of mince was also tested. Response surface methodology (RSM) was used to optimize the temperature and pH conditions to test for the proteases present in *Kudoa*-infected fish mince.

2. 2 Materials and Methods

2.2.1 Chemicals

Trypsin (T 7409), azocasein synthetic substrate (A 2765), L-leucine (L 8912), and 2,4,6-trinitrobenzenesulfonic acid (TNBS) (92822) were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. All other reagents were purchased from Fisher Scientific Co., Ottawa, ON.

2.2.2 Fish samples

Pacific hake were harvested off the coast of Vancouver Island, BC, Canada (around 48.5^{o}N and 124.7-125.5^{o}W) between April and November, 2004. Whole fish (~ 20 fish per week) were transported on ice to the University of British Columbia Food Science Laboratory within two days of capture. About 10-15 g of muscle from the upper nape of each fish was taken to evaluate the intensity of *Kudoa* infection. Fish were individually packed in polyethylene bags, labeled and stored frozen (-25 °C) until used for further analysis.

2.2.3 *Kudoa* spore counts

The method described by Dawson-Coates and others (2003) was modified for isolating and counting of *Kudoa* spores from the muscle samples taken from the upper nape or other locations on individual fish, or from the batches of fish mince prepared from fillets as described in a later section. Thirteen fish with different upper nape spore counts were selected to analyze the distribution of *Kudoa* spores in different parts of the whole fish; for these fish, muscle samples were taken from each area (both right and left sides of fish) as shown in Figure 2.1. Duplicate analyses were performed for each muscle sample.

Briefly, 100 mg of minced muscle sample was weighed into a 15 mL falcon tube and 10.0 mL of trypsin solution (0.04 % w/v in phosphate buffer saline, pH 7.4) was added. The mixture was vortexed, incubated for 1 h in a shaking water bath at 37 ºC, then centrifuged at 2060 g (Beckman GS-6 Centrifuge, Global Medical Instrumentation Inc, Minnesota, U.S.A)

for 15 min at 4 ºC. The supernatant was removed and the pellet containing spores was re-suspended by vortexing well in 1.0 mL phosphate buffer saline. A 20 µL aliquot of the resulting spore suspension or appropriately diluted suspension was taken for *K. paniformis* and *K. thyrsites* spore counting using a hemacytometer. Eight squares in the hemacytometer grid were counted and averaged, and the infection intensity was calculated using the following equation and expressed as number of *K. paniformis*, *K. thyrsites*, or total *Kudoa* spores per 1 g of fish muscle mince.

$$\text{Infection intensity (Spore counts /g mince)} = \frac{\text{Ave. spore count x } 10^4 \text{ (mL}^{-1}\text{) x dilution factor x final volume (mL)}}{\text{Weight of sample used (g)}}$$

2.2.4 Preparation of fish mince

Based on the spore counts of upper nape muscle samples from each fish, 16 batches of fish mince were prepared, composed of fillets from varying numbers of fish per batch (Table 2.1). Size of fish (average weight of whole fish in grams) used in preparing these fillet mince batches are also shown in Table 2.1. Most of the prepared mince batches contained fish captured during different time periods between April and November, 2004. However, batches 1 and 3 were prepared using fish from the same catch, but with different level of infection according to nape spore count results. This was done to check the consistency of proteolytic activity and the texture analysis results among the same catch of fish with different spore levels. Different numbers of fish (2-24) were used in preparing mince batches to check the applicability of results by having different amounts of fish in preparing mince samples for industrial purposes.

Fish were thawed in a cold room (4 °C) for 16 h, filleted then ground twice using a grinder (BEEM Gigant, Butcher & Packer Supply Company, Michigan, U.S.A) with a 4 mm screen. From each batch of fish mince, a sample (~ 100 g) was taken for proteolytic activity assay and *Kudoa* spore counting, and the rest of the mince was portioned (~100 g) into polyethylene bags, vacuum packed and stored frozen (-35 °C) for further analyses.

2.2.5 Determination of pH and temperature optima for endogenous proteases

Response surface methodology (RSM) with a central composite rotatable design was used to optimize temperature and pH conditions for the endogenous proteolytic activity in Pacific hake mince. A fish mince batch with total spore count of 21 x 10^6 *Kudoa* spores per gram mince (Batch number 1 of sixteen batches prepared, Table 2.1) was used for this experiment. During the preliminary experiment (Trial 1, Table 2.2), temperature and pH of proteolytic activity assays were varied between 50-70 °C and 4.0-9.0, respectively. Based on the results obtained from Trial 1, temperature and pH ranges were narrowed down to 50-60 °C and 5.0-7.0, respectively, in the next RSM design (Trail 2, Table 2.2) in order to establish the optimum pH and temperature conditions for endogenous proteases. MINITAB statistical system (Version 12.21, Minitab Inc., State College, Pa, U.S.A) was used for RSM experimental design and data analysis.

2.2.6 Proteolytic activity assay

Fish fillet mince was homogenized manually at 4 °C with 3:1 (w/v) 20 mM phosphate buffer (pH 7.0). The supernatant obtained from the crude homogenate after centrifugation at 17,500 x g for 20 min at 4 °C (SORVALL® RC-5B Plus, Mandel Scientific Co. Ltd., Ontario, Canada) was used immediately to study proteolytic activity using azocasein substrate according to the method described by An and others (1994). Temperature and pH during the assays were set according to the RSM central composite rotatable design (Table 2.2). Briefly, a reaction mixture was prepared containing 2 mg azocasein, 0.625 mL McIlvaine's buffer (0.2 M sodium phosphate, 0.1 M sodium citrate pH 5.5), and 0.375 mL water (to bring the reaction mixture to total volume of 1.0 mL). The pH of the mixture was adjusted according to the Table 2.2 and the mixture was then pre-incubated at the desired temperature for 5 min before addition of 0.25 mL crude homogenate supernatant or control (20 mM phosphate buffer, pH 7.0) to the reaction mixture and incubated for 1 h at the same temperature. After incubation, 0.2 mL cold 50 % (w/v) trichloroacetic acid was added to the mixture to stop the enzymatic reaction, and then the mixture was kept for 15 min at 4 ºC to allow the precipitation of unhydrolyzed proteins. The mixture was centrifuged at 5,700 x *g*

for 10 min (VWR^{TM} Galaxy 16, VWR Scientific Products, Bristol, CT) to separate the supernatant, which contained oligopeptides. The supernatant (0.8 mL) was collected in a new labelled vial. In order to enhance the intensity of the azo color, 60 μL of 10N NaOH was added to the supernatant. Absorbance of the supernatant was read at 450 nm. The protease activity was expressed as ΔA_{450}.

2.2.7 Measuring the extent of autolysis

Eight fish mince samples (from mince batches 1, 4, 5, 7, 8, 10, 12, and 15, Table 2.1) with different *K. paniformis* infection levels were autolyzed (substrate:water ratio = 1:2) for 1 h at the optimum pH and temperature conditions for endogenous proteases present in Pacific hake fish mince according to the RSM study. The extent of protein breakdown during autolysis was estimated by measuring the content of free amino groups at the beginning and the end of hydrolysis, using the TNBS method (Adler-Nissen 1979; Kwan and others 1983). Briefly, a 5.0 mL aliquot was taken from the fish slurry at specific times during hydrolysis, and mixed with 5.0 mL of 24 % trichloroacetic acid. The mixture was centrifuged at 12,100 x g for 5 min. From the supernatant, a 0.2 mL aliquot was mixed with 2.0 mL 0.05 M sodium tetraborate buffer (pH 9.2) and 1.0 mL of TNBS. After incubation in the dark at room temperature for 30 min, 1.0 mL of 2.0 M NaH_2PO_4 containing 18 mM sodium sulfite was added. The absorbance was read at 420 nm (Shimadzu UV-Visible spectrophotometer, Kyoto, Japan). The content of free amino groups was reported as L-leucine equivalents (mequivalents) per gram protein using a standard curve made with L-leucine (0-1.6 mM).

2.2.8 Texture analysis

Fish mince from 16 batches were used to assay textural changes due to endogenous proteolytic activity during cooking. Frozen-stored (-35 °C) fish mince was thawed overnight (16 h) at 4 °C and stuffed into 2.5 cm long and 1.3 cm internal diameter plastic tubes. Each tube contained 3.6 g fish mince, and four tubes were prepared per fish mince. Stuffed tubes were wrapped with food-grade plastic wraps, tied with strings and cooked by placing in a water bath at 52 °C for 15 min and then in a boiling water bath for 5 min. The fish mince reached an internal temperature of 73 ºC. The tubes were cooled in an ice bath for

15 min immediately after cooking and kept at room temperature for another 15-20 min until they reached room temperature (~ 25 °C). At the time of texture analysis using TA-XT2 Texture Analyzer (Stable Micro Systems, Haslemere, UK), plastic wrap was removed from the tube, the cooked meat roll was taken out from the plastic tube and cut into two equal halves (that is, eight samples per fish mince for texture analysis). Freshly-cut surface was used to contact with the 5.0 mm diameter flat-ended probe for texture analysis with a punch test. Maximum force (g) was measured for each sample when the probe penetrated 7.0 mm into the center of the meat roll at a speed of 1.0 $mm \cdot s^{-1}$ (Kim and Park 2000). The average of six consistent measurements (out of eight) was taken as the maximum force value for cooked mince from each batch.

The same procedure was repeated for another set of stuffed mince tubes made with two mince batches (10 and 12, Table 2.1), except that this set was only cooked in a boiling water bath for 20 min, eliminating the pre-incubation step at 52 °C for 15 min as mentioned above.

2.2.9 Statistical analysis

All experiments were performed in triplicate. Statistical analyses were performed using the SPSS statistical program (SPSS 10.0, SPSS Inc., Chicago, IL, U.S.A). Tukey's test was used to evaluate differences among mean values for treatments at $p < 0.05$.

2.3 Results and Discussion

2.3.1 *Kudoa* spore counts

According to spore counts from upper nape muscle samples of individual fish captured during April-November, 2004 in waters near Vancouver Island, BC, the majority of the 80 fish investigated in this study had infection levels between 1×10^6 and 10×10^6 total *Kudoa* spores per gram fish tissue. Further, similar infection levels were observed by analysis in our laboratory of more than 850 fish captured during the summer of 2004.

According to Table 2.1, the total *Kudoa* spore counts per gram of fish mince varied between about 1×10^6 and 300×10^6 among 16 batches. *Kudoa* spores present in fish mince

batches were mainly *K. paniformis*. The presence of a small number of *K. thyrsites* spores (<1 %) was observed in batches 3, 5, 7 and 11, while batch 16 contained about 5 % *K. thyrsites* spores out of total *Kudoa* spores present (Table 2.1). Since *K. paniformis* parasite is reported to be responsible for causing quality problems in Pacific hake (Moran and others 1999), these results indicate that fish with high spore counts captured near Vancouver Island in 2004 would have been at a great risk of post mortem myoliquefaction.

Figure 2.2 shows that total *Kudoa* spore counts of the 16 batches of mince were significantly correlated ($p < 0.05$, $r = 0.983$) with the averaged upper nape spore counts of individual fish used in preparing that particular fish fillet mince batch. Furthermore, spore count values for mince samples were similar to the averaged nape spore count values. Kudo and others (1987) studied the black and white pseudocyst distribution in Pacific hake fillets from fish caught in waters between 37°18´N (near San Francisco) and 48°54´N (west coast of Vancouver Island). According to their observations, more black and/or white pseudocysts were present in the nape than in the tail area of fish. Also, greater incidence of abnormal texture was observed in the nape and dorsal areas of hake (Kudo and others 1987). Several other researchers have also reported a heavier *Kudoa* infection and higher proteolytic activity in the anterior part of the fillet than the posterior direction (Konagaya and Aoki 1981; Tsuyuki and others 1983; Morrissey and others 1995). All these studies counted the pseudocysts in fillets as a measure of infection level. In the present study, however, total *Kudoa* spores were used as a measure of infection level. Since similar spore counts were observed for the averaged nape counts and the mince samples (Figure 2.2), the next experiment was designed to study the distribution of parasites in individual fish by analyzing the spore counts in tissue samples taken from different locations of fish as shown in Figure 2.1. The results obtained for each of the 13 fish analyzed are shown in Table 2.3.

According to Table 2.3, *Kudoa* spores were found in all seven locations of the Pacific hake fish tested in this study. While there were significant differences ($p < 0.05$) in spore counts as a function of sampling region for some of the fish, *Kudoa* spores were generally distributed in all seven sampling regions tested. This confirms the reason for the similar spore counts between averaged nape counts and fish mince prepared using fillets of those fish (Figure 2.2). According to these results, fish mince spore count can be used as an indicator

for the average infection level of Pacific hake fish used to prepare that particular fish fillet mince using the fish caught off the coast of Vancouver Island, Canada.

2.3.2 Determination of pH and temperature optima for endogenous proteases

According to a study conducted by An and others (1994), the optimal conditions for Pacific hake protease activity were pH 5.50 and 55 °C, when tested using a crude fillet homogenate prepared using fish caught off the Oregon coast, WA and using the azocasein synthetic substrate. Porter (1992) reported a similar pH optimum at 5.25 when proteolytic activity was assayed at 55 °C using a crude enzyme preparation from Pacific hake, which were caught near west coast of U.S.A. This study further indicated that the fish caught from different locations can have extreme ranges of enzymatic activity (Porter 1992). This could mainly be due to the type and extent of *Kudoa* infection in fish caught from different locations. The present study used the assay method described by An and others (1994) with synthetic azocasein substrate to measure endogenous protease activity of Pacific hake fish fillet mince homogenate prepared using the fish captured near Vancouver Island. Applicability of literature reported optimal temperature and pH conditions for the Pacific hake fish mince homogenate prepared in this project was studied using the RSM. Further, the azocasein synthetic substrate was used since it has been found to be the most suitable in determining proteolytic activity responsible for Pacific hake protein breakdown, due to its high solubility in the pH range of 5-10 (An and others 1994).

Regression analysis of the data obtained from the RSM experiments (Table 2.2) produced appropriate models in both Trial 1 ($S = 0.03346$, $R^2 = 98.5$ %, lack-of-fit $p = 0.092$) and Trial 2 ($S = 0.03489$, $R^2 = 92.1$ %, lack-of-fit $p = 0.557$). The contour plot from Trial 1 (Figure 2.3a) indicated that the endogenous proteolytic activity of Pacific hake homogenate varied with different temperature and pH combinations, with the optimum conditions between pH 5.0-7.0 and temperature of 50-60 °C. The next RSM experiment (Trial 2, Table 2.2), conducted within this optimal range of conditions, narrowed down the optimum pH and temperature conditions near pH 5.50 and temperature of 52 °C (Figure 2.3b). To confirm this result, another set of experiments were performed using the same azocasein substrate and

narrower temperature and pH ranges of 51-55 °C and 5.25-5.75, respectively (Figure 2.4). The effect of temperature on proteolytic activity was similar at pH 5.25 and 5.50; further, no significant ($p > 0.05$) differences could be seen in proteolytic activity in the range of pH from 5.25 to 5.50 and temperature from 52 to 55 °C, under which conditions the highest proteolytic activities were observed. However, at pH 5.75, a different pattern was observed, with optimum conditions around 53-54 °C.

Based on these results (Figures 2.3 and 2.4), optimum conditions for endogenous enzyme activity in fish fillet mince were concluded to be at pH 5.25-5.50 and temperature of 52-55 °C, which agrees with previously reported literature on Pacific hake protease activity mentioned above. The conditions of pH 5.50 and temperature 52 °C, which were the optimum conditions obtained from RSM experiments, were selected to perform subsequent experiments to analyze proteolytic activities of different fish mince batches prepared, and to autolyze several fish mince batches as explained in the methodology section.

2.3.3 Influence of *Kudoa* spore counts on endogenous proteolytic activity and extent of autolysis

Figure 2.5 shows how the proteolytic activity of fish mince changes with the level of *K. paniformis* spore counts. A strong linear relationship ($r = 0.957$, $p < 0.001$) was observed between spore counts below 10×10^6 per g fish mince and endogenous proteolytic activity. Beyond that infection level, proteolytic activity did not significantly change ($p > 0.05$), except for the extremely infected fish mince with spore count of 308×10^6 per gram mince. Similar strong correlation ($r = 0.960$, $p < 0.001$) was observed between total *Kudoa* spore counts and endogenous proteolytic activity. This could be due to the fact that the fish mince samples prepared using fish caught near Vancouver Island during summer months contained mainly *K. paniformis* spores (Table 2.1). Further, the free amino content of autolyzed fish mince samples having different *K. paniformis* infection levels was significantly correlated ($r = 0.843$, $p = 0.009$) with their proteolytic activity (data not shown). This indicates that the extent of protein breakdown in Pacific hake fish mince at 52 °C and pH 5.50 can be predicted, roughly, by measuring its *K. paniformis* infection level or the proteolytic activity.

These results agree with the literature data on higher proteolytic activity in hake fish infected with *K. paniformis* (Konagaya and Aoki 1981; Patashnik and others 1982; Kudo and others 1987). Seymour and others (1994) suggested that the increased proteolytic activity in infected Pacific hake may be due to an increase in activity and/or in expression of cathepsin L present in fish muscle. Etherington (1984) also reported that cathepsin L, present in lysosomes of macrophages, is expressed at higher levels as a response to infection or infestation. Thus, the relatively consistent and high proteolytic activity in fish infected with more than 10 x 10^6 *K. paniformis* spores per gram tissue may reflect the maximum level of cathepsin L that can be released from muscle tissues due to infection. This infection level (>10 x 10^6 *K. paniformis* spores per gram tissue) is similar to the stage reported in the literature where mature black pseudocysts can be observed in Pacific hake fillets having high proteolytic activity (Morrissey and others 1995). However, counting the *Kudoa* spores, either *K. paniformis* or total spores in this study as the *K. thyrsites* infection was not that significant, gives a better quantitative measurement of infection level compared to the visual culling method.

2.3.4 Influence of *Kudoa* spore counts on cooked texture of Pacific hake fish mince

Figure 2.6 shows how the texture of mince samples changes with *K. paniformis* spore counts after cooking conditions that included 15 minutes at the optimal conditions (52 ºC) for endogenous proteases present in fish. In general, texture values decreased with the increase of spore counts. Change in texture during cooking was significantly and negatively correlated with the proteolytic activity of fish mince ($r = -0.620$, $p = 0.031$), and with the free amino content during autolysis ($r = -0.874$, $p = 0.005$) over the whole range of *K. paniformis* spore counts studied. Fish mince batches 10 and 12 which contained 100 x 10^6 and 300 x 10^6 *K. paniformis* spores per gram mince, respectively, had very soft texture (maximum force < 60 g, Figure 2.6) after cooking. However, it should be noted that the cooking temperature used in this experiment included incubation for 15 minutes at the optimal temperature (52 °C) for the endogenous proteases present in fish. Thus, these results indicate the worst case scenario. Fish mince rolls made using mince batches 10 and 12, and cooked directly in a

boiling water bath for 20 min had texture values of 189 ± 27 g and 172 ± 4 g, respectively. This indicates that rapid cooking methods can be used to avoid prolonged exposure to the optimal temperature condition for the endo-proteases to minimize soft-flesh formation during cooking. Cooking methods such as deep-fat frying or microwaving would be more effective in keeping the desirable cooked texture of Pacific hake compared to boiling or baking (Patashnik and others 1982; Nelson and others 1985). Cathepsin L-like proteases present in muscle tissues can be inactivated by holding the temperature above 70 °C for about 15 min (Nelson and others 1985). Rapidly cooked Pacific hake fillets from the frozen stage (rapid freezing in dry ice at sea, held for 1-2 days in superchilled water and frozen) have been found to retain normal cooked texture quality without any significant evidence of softness (Nelson and others 1985). These storage and cooking methods should minimize textural changes in Pacific hake during further processing. However, relationships between *Kudoa* spore count and the textural quality of fish mince processed using these rapid chilling and cooking methods should be elucidated to give a proper indication of textural changes relative to the spore counts of fish mince.

2.4 Conclusions

According to the results of the present study, offshore Pacific hake caught near Vancouver Island, BC during the summer months of 2004 were mainly infected with *K. paniformis* parasite, which can cause textural changes in hake muscle tissues during post mortem and/or thermal processing. The spore enrichment method developed by Dawson-Coates and others (2003) was successfully applied for enumerating *Kudoa* spores and identifying *K. paniformis* and *K. thyrsites* spores in fish mince batches prepared for further processing, as a quick measurement (indicator) of average infection level. Pacific hake with *K. paniformis* spore counts greater than 10 x 10^6 per gram fish mince possessed high proteolytic activity. Texture values of fish mince samples, after holding at the optimal temperature condition (52 °C) for endogenous proteases, decreased with the increase of *K. paniformis* spore counts. The free amino content during autolysis of fish mince at 52 °C and pH 5.50 was significantly correlated to the proteolytic activity and cooked texture. These

relationships will be quite useful in finding new applications for the huge stock of Pacific hake caught every year in BC waters. Studies are already underway in our laboratory to make fish protein hydrolysates having various functional and bioactive properties from Pacific hake with the help of increased cathepsin L-like protease levels in fish infected with *K. paniformis*.

Table 2.1 *Kudoa* spore counts of sixteen Pacific hake fish mince batches

Batch #	Number of fish in batch	Average weight ± SD[1] of whole fish (g)	Total *Kudoa* spore counts (x10^6/g fish mince)	*K. thyrsites* spore counts (x10^6/g fish mince)	*K. paniformis* spore counts (x10^6/g fish mince)
1	7	1105 ± 411	20	ND[2]	20
2	23	779 ± 266	4	ND	4
3	10	1222 ± 780	4	0.01	4
4	10	671 ± 89	30	ND	30
5	3	879 ± 254	9	0.04	9
6	3	611 ± 15	1	ND	1
7	2	895 ± 398	1	0.006	1
8	2	865 ± 3	7	ND	7
9	2	610 ± 119	3	ND	3
10	2	806 ± 175	100	ND	100
11	2	713 ± 52	4	0.01	4
12	2	736 ± 48	300	ND	300
13	3	557 ± 95	1	ND	1
14	3	686 ± 176	1	ND	1
15	3	681 ± 247	2	ND	2
16	3	925 ± 347	2	0.09	2

[1]SD = Standard deviation

[2]Spores not detected in duplicate analyses under 200x magnification

Table 2.2 RSM central composite rotatable designs to determine optimal pH and temperature for endogenous proteolytic activity in Pacific hake homogenate

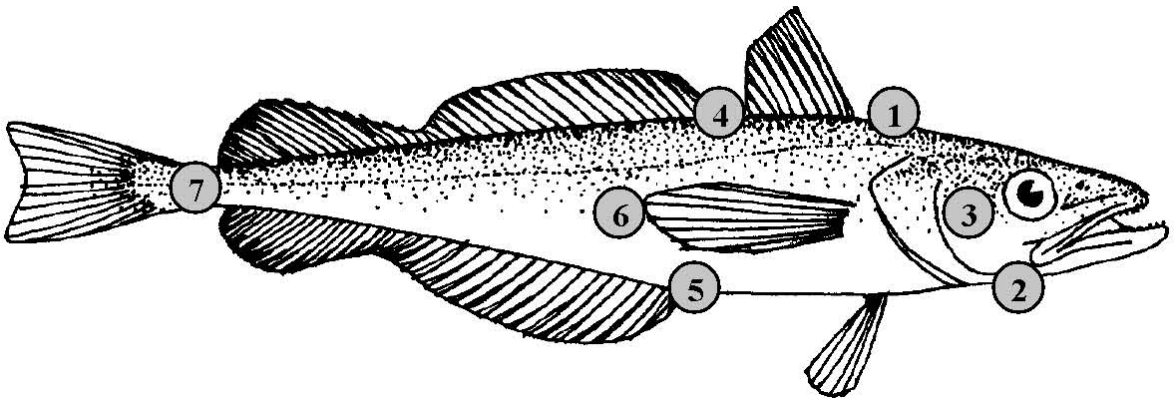
	Trial 1		Trial 2	
Run order	**pH**	**Temperature,°C**	**pH**	**Temperature,°C**
1	6.50	45.9	6.00	47.9
2	9.00	70.0	7.41	55.0
3	6.50	60.0	5.00	60.0
4	6.50	60.0	6.00	55.0
5	6.50	60.0	5.00	50.0
6	2.96	60.0	4.58	55.0
7	6.50	74.1	6.00	62.1
8	6.50	60.0	6.00	55.0
9	10.04	60.0	6.00	55.0
10	6.50	60.0	7.00	50.0
11	9.00	50.0	7.00	60.0
12	4.00	70.0	6.00	55.0
13	4.00	50.0	6.00	55.0

Table 2.3 Distribution of *Kudoa* spores in 13 Pacific hake fish as a function of 7 sampling locations on the whole fish[1]

Fish #	Total *Kudoa* spore counts (x10^6 /g fish tissue) of tissue samples taken from different locations of individual fish[2]						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1	2^{a}	1^{a}	1^{a}	0.7^{a}	1^{a}	2^{a}	0.5^{a}
2	0.3^{a}	0.6^{ab}	0.2^{a}	1^{b}	0.4^{ab}	0.6^{ab}	0.8^{ab}
3	30^{c}	10^{ab}	30^{bc}	30^{bc}	10^{ab}	30^{c}	5^{a}
4	30^{cd}	6^{a}	10^{ab}	30^{bc}	10^{a}	40^{d}	8^{a}
5	0.8^{b}	0.3^{a}	0.3^{a}	0.3^{a}	0.3^{a}	0.4^{a}	0.3^{a}
6	$70^{a,b}$	30^{a}	50^{a}	70^{a}	100^{b}	60^{a}	30^{a}
7	0.4^{a}	0.4^{a}	0.4^{a}	0.3^{a}	0.4^{a}	0.2^{a}	0.2^{a}
8	0.3^{a}	0.3^{a}	0.2^{a}	0.2^{a}	0.2^{a}	0.3^{a}	0.2^{a}
9	7^{bc}	2^{a}	2^{a}	6^{b}	9^{c}	3^{a}	2^{a}
10	1^{ab}	2^{c}	$1^{a,b}$	1^{ab}	1^{ab}	1^{bc}	0.8^{a}
11	200^{abc}	90^{a}	100^{ab}	200^{abc}	300^{bc}	300^{c}	40^{a}
12	100^{b}	30^{a}	40^{a}	100^{ab}	100^{b}	100^{b}	80^{ab}
13	100^{b}	70^{ab}	50^{a}	100^{b}	100^{b}	200^{c}	10^{a}

[1]*Kudoa* spores were enumerated using tissues from 7 sampling locations taken from both sides of each fish, as shown in Figure 2.1

[2]Means of duplicate samples taken from both sides of fish (that is, n = 4). Means with the same letter in a row are not significantly different, $p > 0.05$



1 = Medial dorsal somatic (nape), 2 = Hyohyoideus ventralis, 3 = Adductor mandibular muscles, 4 = Medial dorsal (posterior to dorsal fin), 5 = Ventral medial somatic, 6 = Lateral fillet, 7 = Fin

Figure 2.1 Locations of seven tissue sampling sites to study *Kudoa* spore distribution in Pacific hake

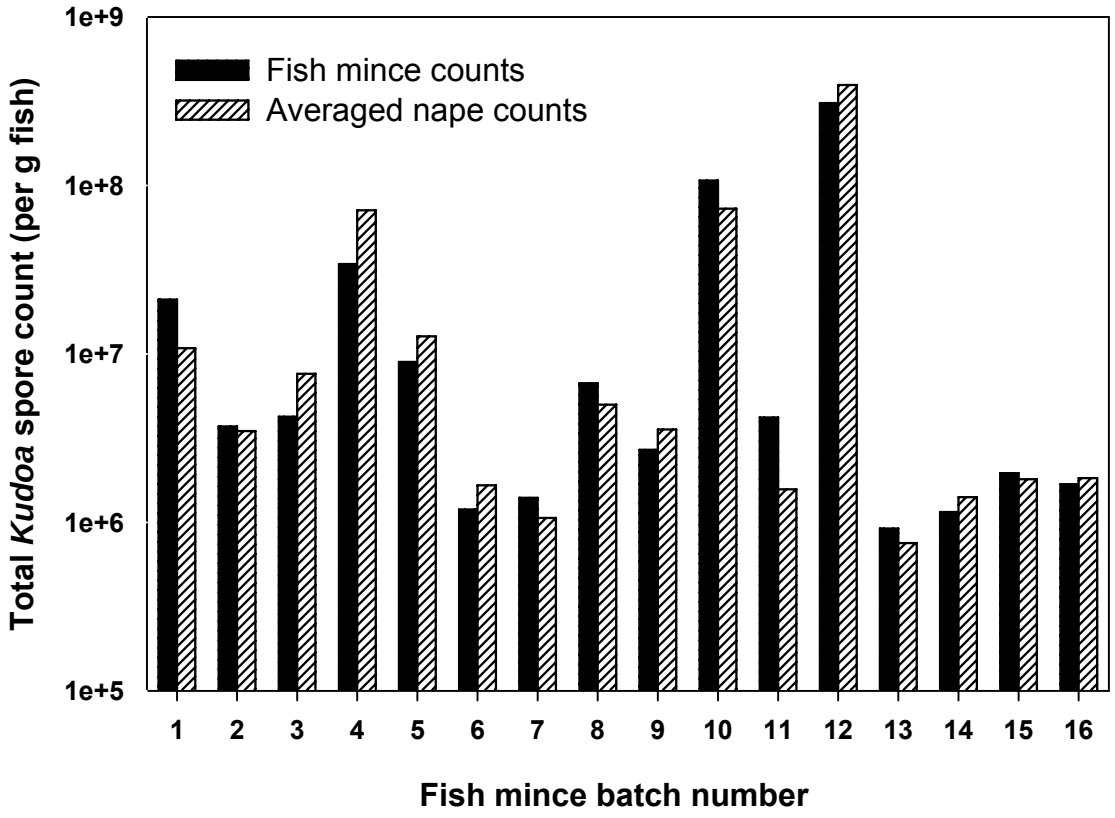
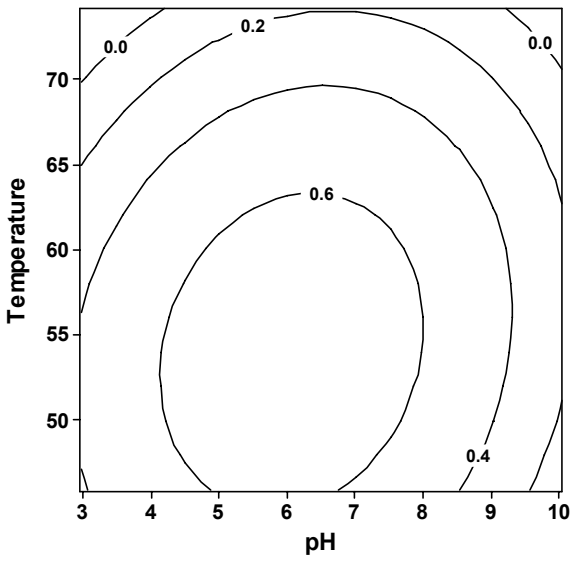


Figure 2.2 Comparison of total *Kudoa* spore counts of fish mince to the averaged medial dorsal somatic (nape) spore counts of individual fish pooled to prepare mince batches.

(a)



(b)

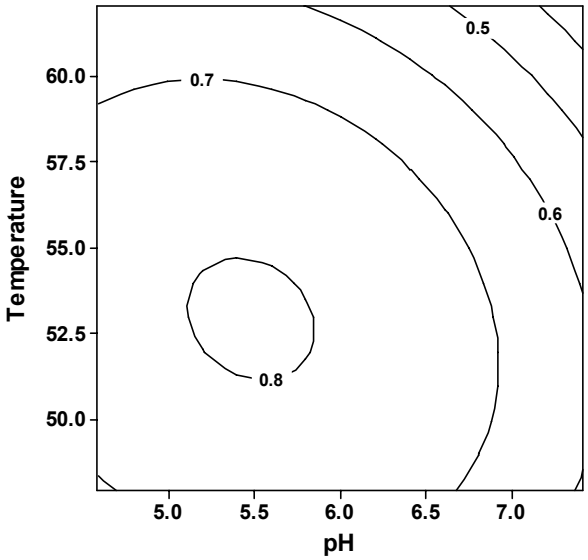


Figure 2.3 Contour plots from RSM Trials 1 (a) and 2 (b) showing optimum pH and temperature (°C) conditions for endogenous proteases present in Pacific hake fish fillet mince. Contour lines represent different absorbance values at 420 nm from the proteolytic activity assay using azocasein substrate.

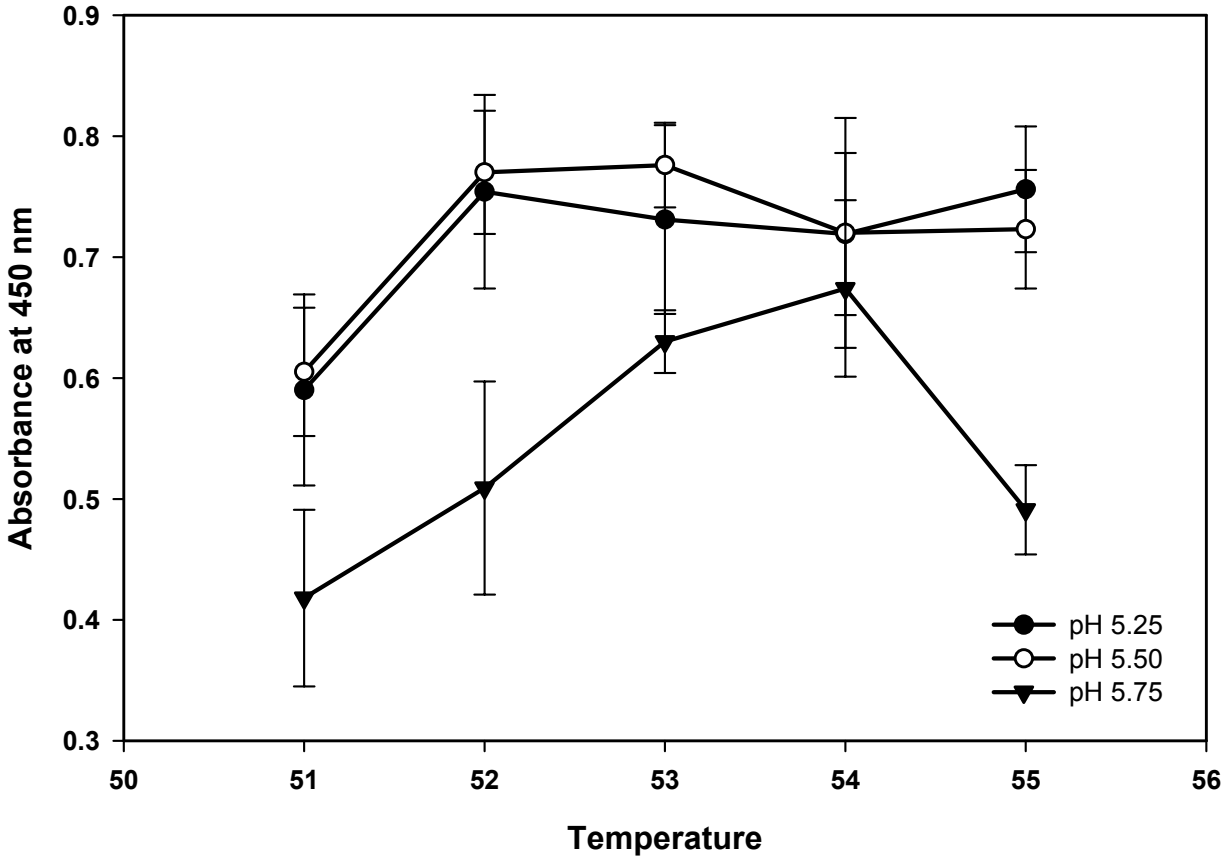


Figure 2.4 Change in endogenous proteolytic activity of fish fillet mince with temperature (°C) and pH.

mean values of n = 3; bars = standard deviation.

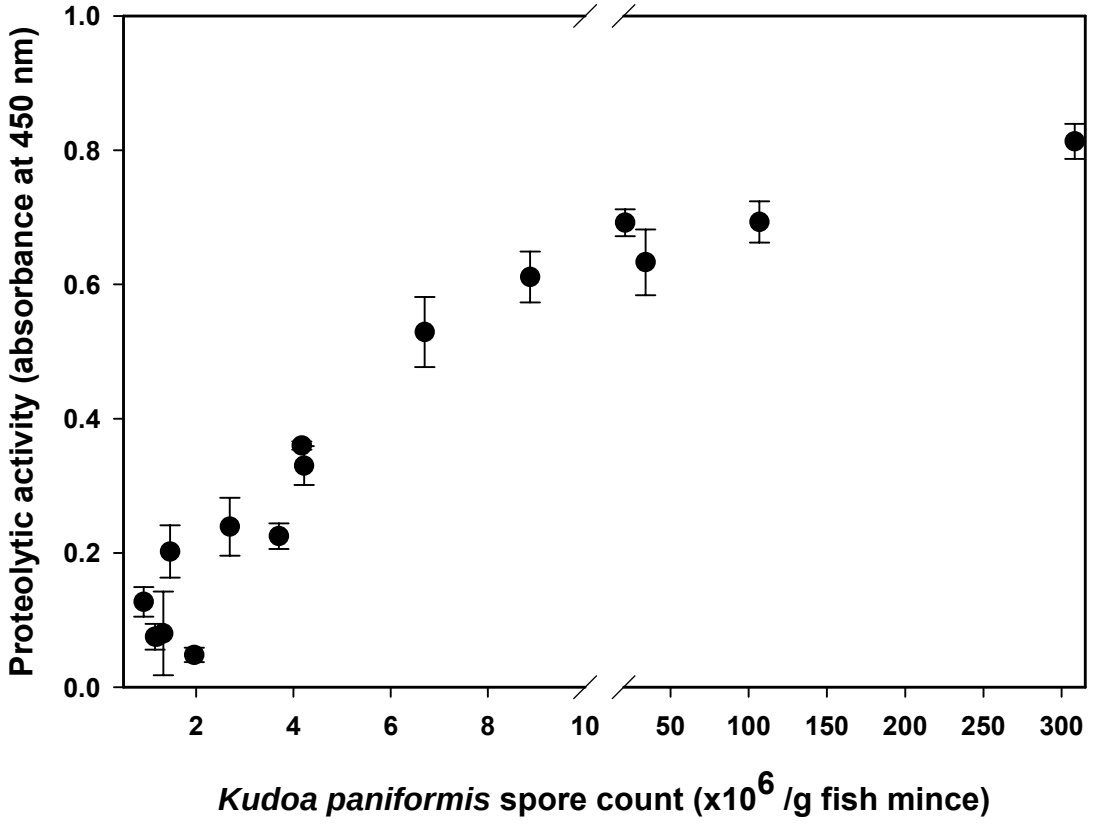


Figure 2.5 Variation of proteolytic activity with number of *Kudoa paniformis* spores present in fish mince.

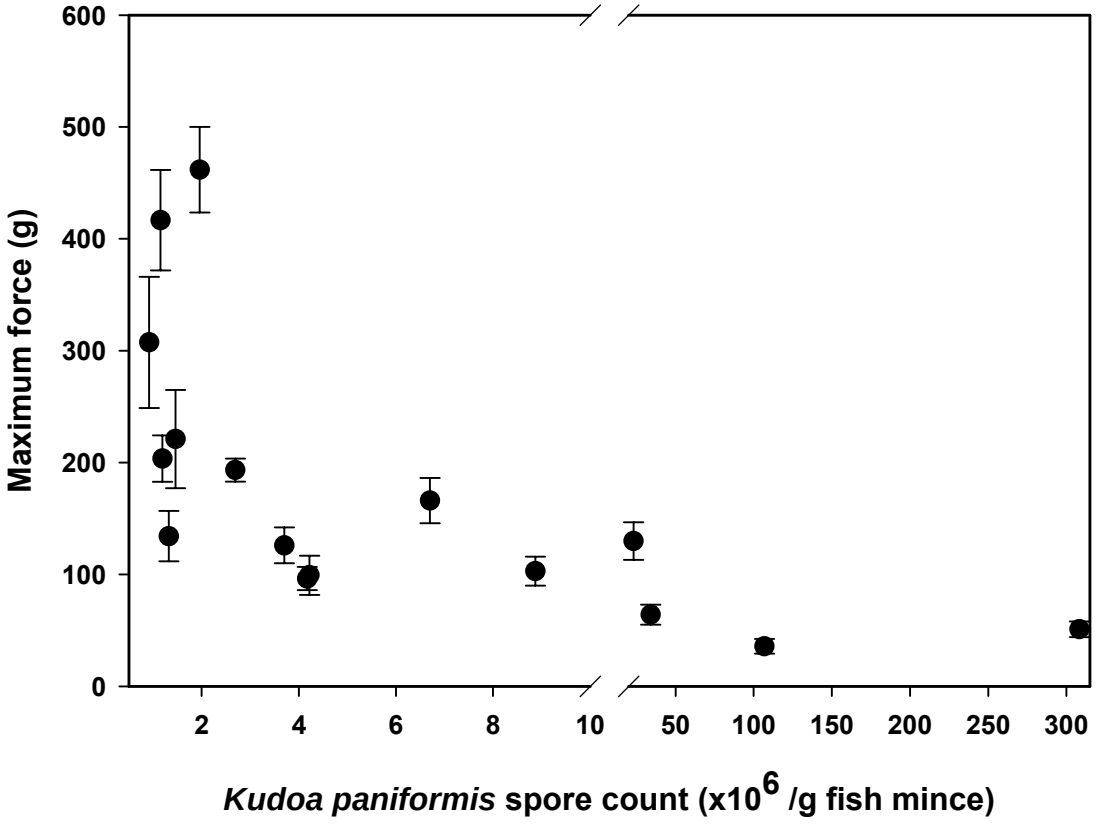


Figure 2.6 Texture (measured as maximum force, g) of cooked Pacific hake fish mince as a function of *Kudoa paniformis* spore counts.

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CHAPTER 3 Autolysis-assisted Production of Fish Protein Hydrolysates with Antioxidant Properties from Pacific Hake (*Merluccius productus*)[1]

3.1 Introduction

Dietary proteins have been found to play a significant role in improving human health beyond their well recognized nutritional value (Hartmann and Meisel 2007). Fish protein hydrolysates (FPH) have been reported to possess antioxidative, antihypertensive, antimicrobial and immunomodulatory properties (Shahidi and others 1995; Fujita and Yoshikawa 1999). In particular, protein hydrolysates with antioxidant properties have become a topic of great interest for pharmaceutical, health food, as well as food processing/preservation industries (Alasalvar and others 2002; Hagen and Sandnes 2004). The bioactive molecules in FPH responsible for these properties are peptides that are released upon hydrolysis of fish proteins, by the enzymes already present in fish mince (endogenous) and/or by different enzymes added at appropriate levels to the fish mince (exogenous). Antioxidant activity has been reported for protein hydrolysates prepared from various fish sources such as capelin, tuna, mackerel, yellowfin sole, Alaska pollack, Atlantic salmon, hoki, conger eel, and scad (Amarowicz and Shahidi 1997; Jao and Ko 2002; Wu and others 2003; Jun and others 2004; Berge 2005; Je and others 2005a; Je and others 2005b; Ranathunga and others 2006; Thiansilakul and others 2007). Further, a brine solution containing salmon FPH injected into smoked salmon fish fillets was shown to reduce lipid oxidation measured as 2-thiobarbituric acid reactive substances (TBARS) during 6 weeks of cold storage (4 °C) and 8 months of frozen storage (-18 °C) (Hagen and Sandnes 2004).

Pacific hake (*Merluccius productus*), also referred to as Pacific whiting, is a fish with low economic value because of the presence of a parasitic Myxosporea, *Kudoa paniformis*,

[1]A version of this chapter has been published. Samaranayaka AGP, Li-Chan ECY. 2008. Autolysis-assisted Production of Fish Protein Hydrolysates with Antioxidant Properties from Pacific Hake (*Merluccius productus*). Food Chem 107: 768-776.

and the ‘‘soft flesh” condition that results from autolysis during subsequent processing (Kabata and Whitaker 1985). Increased cathepsin L-like endogenous proteolytic activity of *K. paniformis* infected Pacific hake muscle (An and others 1994) could be an asset, rather than a disadvantage, in making FPH without the need for adding commercial enzymes. Optimum conditions for the proteolytic activity of Pacific hake fish fillet mince were pH 5.25–5.50 and 52–55 °C (An and others 1994, Samaranayaka and others 2006 and Chapter 2). *K. paniformis* spore count was significantly ($p< 0.05$) correlated with endogenous proteolytic activity of fish mince, and Pacific hake with *K. paniformis* spore counts greater than 1×10^7 per gram fish mince possessed high proteolytic activity (Chapter 2).

Benjakul and Morrissey (1997) showed the feasibility of preparing FPH with good nitrogen recovery and comparable amino acid composition to fish muscle by hydrolyzing Pacific whiting solid wastes using the commercial enzyme Alcalase® 2.4L. Further, endogenous fish enzymes were successfully used in preparing fish sauce with various biochemical properties from Pacific whiting and its surimi byproducts (Tungkawachara and others 2003), as well as from other fish sources such as capelin (Shahidi and others 1995). However, the endogenous protease activity was attributed predominantly to digestive enzymes of the viscera (Shahidi and others 1995). To date, there has not been any investigation on producing FPH by autolysis of Pacific hake fish muscle, nor on the possible influence of *Kudoa* parasitization on production and antioxidant properties of the FPH.

The overall objective of this study was to investigate the potential of using endogenous enzymes present in Pacific hake fish muscle to produce FPH with antioxidative properties. Firstly, the antioxidant properties of the FPH produced by autolysis as a function of hydrolysis time were studied, and compared to FPH produced by addition of food-grade commercial enzymes, Flavourzyme® 500L and Validase® bacterial neutral protease (BNP). Secondly, the effect of *K. paniformis* infection level of fish mince on autolysis-assisted production of FPH with antioxidant properties was investigated. Antioxidant activity of FPH was assessed using in vitro chemical assays and model systems, compared to the activities of two common synthetic food antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), and the natural antioxidant, α-tocopherol. The development of FPH ingredients with antioxidative properties for food and nutraceutical applications could be an

ideal approach to utilize the huge stock of Pacific hake caught in waters near the Pacific coast.

3.2 Materials and Methods

3.2.1 Materials

Validase® BNP (bacterial neutral protease from *Bacillus subtilis*, 2000 NPU/g) in liquid form was a gift from Valley Research Inc., South Bend, IN. Flavourzyme® 500L (protease from *Aspergillus oryzae*, 500 LAPU/g, product of Novozymes®) was kindly donated by Brenntag Canada Inc., Langley, BC. Trypsin (T7409), L-leucine (L8912), 2,4,6-trinitrobenzenesulphonic acid (TNBS) (92822), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (11557), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (238813), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (D9132), potassium ferricyanide (455946), ferrozine (P9762), ferrous chloride (220299), fluorescein (F6377), 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) (440914), linoleic acid (L1376), BHT (W218405), and α-tocopherol (T4389) were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. BHA (101159) was purchased from ICN Biomedicals Inc., Aurora, Ohio.

3.2.2 Fish samples and preparation of fish mince

Pacific hake were harvested off the coast of Vancouver Island, Canada (48.5°N 124.7–125.5°W) between April 2004 and May 2005. Whole fish were transported on ice to the University of British Columbia Food Science Laboratory within two days of capture, individually packed in polyethylene bags, labeled and stored frozen (-25 °C) until used. Based on the *K. paniformis* spore counts determined as described in the next section, fillets from 34 selected fish were used to prepare fish mince batches with infection level of 0 (Inf-0), 1 (Inf-1), 2 (Inf-2), 7 (Inf-7), 9 (Inf-9), 20 (Inf-20), 30 (Inf-30), and 100 (Inf-100) x 10^6 spores/g mince. Weights of fish used ranged between 434 and 1946 g. Fish were thawed in a cold room (4 °C) for 16 h, filleted, de-skinned, and then ground twice using a grinder (BEEM Gigant, Butcher and Packer Supply Company, Detroit, MI) with a 4 mm screen. From each

batch of fish mince, a sample (~100 g) was taken for *K. paniformis* spore counting and proximate analysis, and the rest of the mince was portioned (~100 g) into polyethylene bags, vacuum packed and stored frozen (-35 °C) for further analyses.

3.2.3 *K. paniformis* spore counts

The method of Dawson-Coates and others (2003), as modified by Samaranayaka and others (2006, Chapter 2), was used for isolating and counting *K. paniformis* spores in the batches of fish mince prepared. Duplicate analyses were performed for each mince sample, and the results were expressed as number of *K. paniformis* spores per g of sample.

3.2.4 Proximate analysis

Proximate composition of Pacific hake fish fillet mince was determined by analysis of Inf-20 batch. Moisture and ash contents were analyzed using standard methods of the Association of Official Analytical Chemists (AOAC) 930.15 and AOAC 942.05, respectively (AOAC, 1995). Lipid content was determined by the method of Bligh and Dyer (1959), and crude protein was determined from nitrogen content (N x 6.25) measured by a combustion method AOAC 992.15 (AOAC 1995) using a Leco instrument (Leco Crop., St. Joseph, MI) calibrated with ethylenediaminetetraacetic acid (EDTA) (9.58 %N).

3.2.5 Preparation of FPH

In the first part of the study, several FPH were prepared using fish mince from Inf-20 batch, by incubation at pH 5.50 and 52 °C as shown in Figure 3.1. Autolysis of the fish mince slurry for 1-6 h was used to prepare E-1h, E-2h, E-3h, E-4h, and E-6h FPH. Food grade commercial enzymes Validase® BNP (V) and Flavourzyme® 500L (F) were used to make V-2h, F-2h, E+V- 2h, and E+F-2h FPH (enzymes added at 2 % (v/w) level of the protein content of the fish mince). Two types of control FPH were also prepared. The E-0h FPH was a control to measure the extent of autolysis during the 10-12 min incubation in the 55 °C water bath required to bring the slurry temperature to 52 °C before starting the actual hydrolysis process by adjusting pH to 5.50. The second type of control (C-2h) was prepared

by inactivating endogenous proteases prior to incubation by heating fish mince slurries in a boiling water bath (>90 °C) for 15 min. Another set of FPH was prepared following a similar procedure shown in Figure 3.1, using Inf-30 fish mince and autolysis or Validase® BNP, and also including E-5h, C-0h, C-1h, V-1h, and E+V-1h FPH.

In the second part of the study, C-0h, E-1h, and E-6h FPH were made from each of the eight fish mince batches in order to assess the effect of fish mince *K. paniformis* infection level on production of FPH with antioxidative properties.

After incubation for the indicated time of autolysis with endogenous enzyme and/or hydrolysis with Validase or Flavourzyme, 100 mL aliquots of the resulting FPH and control slurries were heated in a boiling water bath (>90 °C) for 15 min, centrifuged at 17,000 x g for 20 min, and filtered through 2 layers of cheese cloth. The filtrates were adjusted to pH 7.0 and freeze dried. Freeze dried FPH samples were homogenized using a pestle and a mortar, then stored in sealed vials at -18 °C until used for further analysis. Yields of FPH produced were calculated using the following equation.

$$\text{FPH yield}\,(\%, \text{dry basis}) = \frac{\text{Weight of freeze dried FPH (g)}}{\text{Dry matter weight of fish mince used (g)}} \times 100$$

3.2.6 Measurement of the extent of hydrolysis

The extent of hydrolysis was estimated by measuring the content of free amino groups using the TNBS method (Adler-Nissen 1979) as described in Chapter 2 (Section 2.2.7) and expressed as L-leucine equivalents (mmol)/g protein of the fish mince slurry after hydrolysis, based on a standard curve constructed with 0 - 1.6 mM L-leucine.

3.2.7 Measurement of antioxidative activity of FPH

3.2.7.1 DPPH radical scavenging capacity assay

The method described by Kitts and others (2000) was used with slight modifications in order to assess the DPPH radical scavenging activity of FPH. DPPH solution (1.8 mL, 0.1 mM in 80 % ethanol) was mixed with FPH solution (0.20 mL, at 3 mg/mL final assay concentration in 50 % ethanol). Absorbance (Abs.) of the solution was read at 517 nm after

30 minutes of incubation at room temperature. For the assay control, 0.20 ml of 50 % ethanol was used in the assay instead of the FPH solution. Sample controls were also made for each FPH by mixing 0.20 ml FPH solution with 1.8 mL of 80 % ethanol. Radical scavenging capacity of FPH was calculated as follows:

$$\text{DPPH radical scavenging capacity}(\%) = \left(1 - \frac{(\text{Abs. of sample - Abs. of sample control})}{\text{Abs. of assay control}}\right) \times 100$$

3.2.7.2 Ferric ion reducing antioxidant capacity

The method described by Oyaizu (1988) was used to measure the ferric ion reducing capacity of FPH. A 2.0 mL aliquot of FPH stock solution in phosphate buffer (PB) (0.2 M, pH 6.6) was mixed with 2.0 mL of the same buffer and 2.0 mL of 1 % potassium ferricyanide to yield final FPH concentration of 3 mg/mL. After incubation at 50 °C for 20 min, 2.0 mL of 10 % trichloroacetic acid was added, and a 2.0 mL aliquot was mixed with 2.0 mL of distilled water and 0.4 mL of 0.1 % ferric chloride. Absorbance at 700 nm after 10 minutes was measured as an indication of reducing power.

3.2.7.3 Trolox equivalent antioxidant capacity (TEAC) assay

A modified method of Re and others (1999) was used to evaluate the ABTS radical scavenging capacity of FPH. A stock solution of ABTS radicals was prepared by mixing 5.0 mL of 7 mM ABTS solution with 88 μL of 140 mM potassium persulfate, and keeping in the dark at room temperature for 16 h. An aliquot (0.5 mL) of stock solution was diluted (~ 40 mL) with PB (5 mM, pH 7.4) containing 0.15 M NaCl in order to prepare the working solution of ABTS radicals with absorbance at 734 nm of 0.70 ± 0.02. A 65 μL aliquot of FPH dissolved in the same PB (66.67 μg/mL final assay concentration) or only buffer (for the control) was mixed with 910 μL of ABTS radical working solution, incubated for 8 min at room temperature in the dark, and then absorbance was measured at 734 nm. The % reduction of $ABTS^{+}$ to ABTS was calculated according to the following equation:

$$\text{ABTS radical scavenging capacity}(\%) = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Further, the TEAC, which is the concentration of sample giving the same % inhibition of absorbance at 734 nm of ABTS radical cation as 1 mM Trolox, was determined by assessing ABTS radical scavenging capacities of FPH solutions at four different final assay concentrations (6.67, 16.67, 33.33, and 66.67 μg/mL) and by using a standard curve prepared with 0-20 μM Trolox final assay concentrations. TEAC values for BHT and BHA were obtained using a similar procedure with the appropriate sample concentrations.

3.2.7.4 Oxygen radical absorbing capacity (ORAC) assay

ORAC of selected FPH was measured using the method described by Kitts and Hu (2005). Briefly, the samples and Trolox antioxidant standard in PB (50 mM, pH 7.0) were incubated with 60 nM fluorescein at 37 °C for 15 min in a 96-well plate (FluoroNunc™ Fluorescent microplate, VWR International, Ltd., Mississauga, ON). After addition of the peroxyl radical initiator, AAPH, the fluorescence using excitation wavelength of 485 nm and emission wavelength of 527 nm was continuously recorded for 60 min (Fluoroskan Ascent FL, Thermo Fisher Scientific, Inc., Milford, MA). Data transformation and interpretation was performed according to the method described by Valos and others (2004). The ORAC value was expressed as μmol Trolox equivalents/g sample.

3.2.7.5 Metal ion chelating activity

The Fe^{2+} chelating activity of FPH at 5 mg/mL assay concentration was measured by the method of Decker and Welch (1990). An FPH stock solution made with deionized water (1.0 mL) was mixed with 3.7 mL of deionized water and 0.1 mL of ferrous chloride (2 mM) such that the concentration of FPH in the solution mixture was 3 mg/mL. The mixture was incubated at room temperature for 30 min and reacted with 0.2 mL ferrozine (5 mM) for another 10 min. The absorbance of the mixture (formation of the ferrous ion-ferrozine complex) was measured spectrophotometrically at 562 nm. For the control, 1.0 mL of deionized water was used in the procedure above instead of the FPH stock solution. The Fe^{2+} chelating effect of FPH was calculated as:

$$Fe^{2+}\text{ ion chelating ability}(\%) = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

3.2.7.6 Lipid peroxidation in a linoleic acid model system

The method described by Osawa and Namiki (1985) was used to measure the inhibition of lipid peroxidation in a linoleic acid/ethanol/water emulsion system by selected FPH samples, BHA, BHT, and α-tocopherol at final assay concentration of 0.2 mg/mL. For FPH, a 5 mg sample was first dissolved in 10.0 mL of PB (50 mM, pH 7.0) and added to a solution of 0.13 mL of linoleic acid and 10.0 mL of absolute ethanol in 250 mL conical flask. BHA, BHT and α-tocopherol were first dissolved in 10.0 mL absolute ethanol and then mixed with 0.13 mL linoleic acid and 10.0 mL of the same PB. The total volume of each flask was adjusted to 25 mL with distilled, deionized water. A control reaction mixture was also prepared using the same procedure, but without adding any FPH sample or commercial antioxidant to the emulsion system. Contents of the flasks were mixed well, and the flasks were sealed and were incubated at 40 °C in a shaking incubator (in the dark) for seven days. Extent of lipid peroxidation was measured by the ferric thiocyanate method (Mitsuda and others 1966) using 0.1 mL aliquots taken in duplicate from each flask at 18 h, 42 h, 90 h, and 162 h of incubation. Briefly, the reaction mixture (0.1 mL) was mixed with 75 % ethanol (4.7 mL), 30 % ammonium thiocyanate (0.1 mL), and 20 mM ferrous chloride solution in 3.5 % HCl (0.1 mL). After 3 min, the absorbance of the colored solution was measured at 500 nm. Reduction of absorbance at 500 nm of emulsion systems containing FPH or commercial antioxidants compared to the control emulsion in the assay indicates its antioxidant potential.

3.2.8 Statistical analysis

All analyses were performed in triplicate. Statistical analyses were performed using the SPSS statistical program (SPSS 10.0, SPSS Inc., Chicago, IL). Tukey's test was used to evaluate differences among mean values for treatments at $p< 0.05$.

3.3 Results and Discussion

3.3.1 Fish mince proximate composition

The proximate composition of Inf-20 fish mince was: 82.99 ± 0.05 % moisture, 15.16 ± 0.06 % crude protein, 1.20 ± 0.03 % ash, and 1.78 ± 0.05 % crude lipids. Similar values (84.62 ± 0.28 % moisture, 14.16 ± 0.61 % crude protein, 1.00 ± 0.10 % ash, and 0.39 ± 0.11 % crude lipid) have been reported previously for muscles of Pacific whiting caught around Oregon, WA (Benjakul and Morrissey 1997).

3.3.2 Antioxidant activity of FPH produced by autolysis and/or exogenous enzyme hydrolysis

The main purpose of this first study using Inf-20 and Inf-30 fish mince was to assess whether antioxidative FPH can be prepared using parasitized Pacific hake fish fillet mince, and particularly to investigate whether addition of exogenous enzymes is necessary to produce FPH with high antioxidant potential compared to the FPH made through autolysis. Two batches of fish mince were used to assess consistency of the results obtained, and the effects of incubation time on the yields, extent of hydrolysis and antioxidant properties were monitored. Validase® BNP and Flavourzyme® 500L were selected as exogenous enzymes since the optimal temperature and effective pH ranges of these two commercial enzymes (i.e., pH 5.5-8.0 and 50-55 °C for Validase® BNP (Valley Research Inc. 2004), and pH 5.0-7.0 and 50 °C for Flavourzyme® 500L (Novozymes® 2004)) are close to the optimal temperature and pH ranges reported for cathepsin L-like endogenous proteases present in Pacific hake i.e., pH 5.25–5.50 and 52–55 °C (Samaranayaka and others 2006 and Chapter 2). Therefore, the same pH and temperature conditions could be used for making FPH by autolysis or exogenous enzymes (Figure 3.1). Validase® BNP is an endopeptidase whereas Flavourzyme® 500L is a mixture of endo- and exopeptidases (Valley Research Inc. 2004, Novozymes® 2004). Cathepsin L, the most active endogenous enzyme in Pacific hake fish muscle at pH 5.5 and 55 °C, is also an endopeptidase (Kang and Lanier 2000). Protein

hydrolysates made using these different enzymes most likely possess peptides of differing lengths and amino acid sequences that may determine their antioxidant capacities.

The yields, extent of hydrolysis measured as free amino group content, and results from different antioxidant assays of FPH are shown in Table 3.1. E+V-2h and E+F-2h, which were made by hydrolyzing fish proteins with both endogenous and exogenous enzymes, had the highest yields (64.2 % and 63.8 %, dry basis, respectively) as well as highest content of free amino groups indicating greater extent of hydrolysis. Yields of FPH made through autolysis ranged from 47.74 % (E-1h) to 55.40 % (E-2h) and did not show large increases after 1 h. Further, similar extents of autolysis were observed for FPH samples made using Inf-20 and Inf-30 fish mince. As expected, control samples of C-0h, C-1h, C-2h and E-0h had undergone the lowest extent of hydrolysis indicating the inactivation of endogenous proteases during initial heating of these samples above 90 °C for 15 min.

For both FPH made using Inf-20 and Inf-30 fish mince, E-1h FPH possessed the highest ($p < 0.05$) DPPH radical scavenging power (Table 3.1). Autolysis beyond 1h reduced the DPPH radical scavenging ability of FPH. Furthermore, FPH with high extent of hydrolysis, such as E+V-2h and E+F-2h, also possessed low DPPH radical scavenging power (Table 3.1). Decrease in antioxidant capacity with higher extent of hydrolysis was previously reported for FPH from mackerel fish fillet mince, in which both peptide content of FPH and the antioxidant potential in a linoleic acid autoxidation system were decreased with hydrolysis beyond 10 h using Protease N (Wu and others 2003). Further, extensive hydrolysis (i.e., 67 % degree of hydrolysis) of yellowfin sole frame protein with mackerel intestine crude enzyme also resulted in decrease of antioxidative activity of FPH in a linoleic acid peroxidation system (Jun and others 2004), and similarly DPPH radical scavenging ability of tuna cooking juice hydrolyzed with Protease XXIII also decreased beyond 2.5 h of hydrolysis (Jao and Ko 2002).

Peptide solubility in a non-polar or an emulsion system can be decreased either by reducing the peptide chain length or by reducing the content of hydrophobic amino acids present in the peptide sequences (Saiga and others 2003). On the other hand, decrease of antioxidative activity of FPH with increasing hydrolysis time may also be due to breakdown of antioxidative peptide sequences formed during early stages of the hydrolysis process. Antioxidative peptides identified from fish sources in literature were reported to have

molecular weights between 500 and 1500 Da (Wu and others 2003; Jun and others 2004; Je and others, 2005a; Je and others 2005b; Ranathunga and others 2006). Many antioxidative peptides identified include hydrophobic amino acid residues valine, or leucine at the N-terminus of the peptides and proline, histidine, or tyrosine in the sequences (Uchida and Kawakishi 1992; Chen and others 1995).

No significant differences ($p > 0.05$) were observed for DPPH radical scavenging capacities between E+V-2h and E+F-2h, as well as between V-2h and F-2h (Table 3.1). Preliminary experiments further indicated that there were no significant differences between these hydrolysates in terms of their reducing capacity as well as the iron chelating ability (results not shown). Further, as mentioned previously, Validase® BNP is an endopeptidase whereas Flavourzyme® 500L is a mixture of endo- and exopeptidases (Valley Research Inc. 2004, Novozymes® 2004). The hydrolysates produced with Flavourzyme® will therefore contain high amount of free amino acids rather than peptides we are interested in producing. It was therefore decided not to further analyze E+F-2h and F-2h hydrolysates during the present study.

All FPH made through 1-6 h autolysis showed high antioxidative potential in the TEAC assay by scavenging ABTS radicals at 66.67 μg/mL final assay concentration (Table 3.1). Compared to the DPPH assay which was conducted in ethanolic solution, TEAC assay was performed in an aqueous medium, and an increase in peptide solubility in aqueous medium with hydrolysis can be one reason for the increase in radical scavenging ability of FPH such as E-6h in the TEAC assay. Considering FPH made with Inf-30 fish mince using Validase® BNP only, antioxidant activity measured as DPPH and ABTS radical scavenging ability as well as the reducing power decreased significantly ($p < 0.05$) during hydrolysis from 1 to 2 h (i.e., V-1h to V-2h) (Table 3.1). When considering FPH made through autolysis, no significant differences ($p > 0.05$) were observed for ferric ion reducing capacity of E-0h, E-1h, and E-2h FPH. However, the reducing power did decrease further when the autolysis time was extended up to 6 h (Table 3.1). V-1h FPH possessed the highest ($p < 0.05$) reducing potential out of all FPH tested. According to these results, FPH samples including E-1h, E-2h, and V-1h possibly contained peptides which functioned as electron donors and could react with free radicals in polar as well as non-polar systems to form more stable products and terminate radical chain reactions.

Some proteins and peptides can chelate metal ions like Fe^{2+}, which can catalyze the generation of reactive oxygen species that accelerates lipid oxidation (Sarkar 1987). Carboxyl and amino groups in the side chains of acidic and basic amino acids are thought to play an important role in chelating metal ions (Saiga and others 2003). At 5 mg/mL assay concentration, the control FPH (C-1h and C-2h) possessed the highest ($p< 0.05$) Fe^{2+} ion chelating ability; FPH made through autolysis did not have high ion chelating ability at the concentrations tested, with the highest ($p< 0.05$) chelating power of only 24 % observed for E-4h (Table 3.1).

The results from this study suggest that antioxidative FPH can be produced using Pacific hake fish fillet mince. Further, it was clear that addition of commercial proteases of Validase® BNP and Flavourzyme® 500L at 2 % level, with or without endogenous muscle proteases, did not contribute in producing FPH with higher antioxidative potential compared to E-1h.

3.3.3 Antioxidative activity of FPH produced from fish mince with varying infection level

About 1000 fish samples received in our laboratory during the period from April, 2004 to May, 2005 were analyzed for *Kudoa* infection level. The majority of these fish samples were infected with *K. paniformis*, with average infection level of 5-20 fish captured per week in the range of 10^6 to 10^7 spores/g mince, and up to 10^8 in some weeks. For the purpose of assessing the effect of fish mince infection level (i.e. endogenous proteolytic activity) on production of FPH with antioxidant activity, fish with different infection levels were selected in order to prepare fish fillet mince batches with *K. paniformis* infection levels between 0 and 100 x 10^6 spores per g mince.

3.3.3.1 Extent of hydrolysis and yields of FPH

Extents of hydrolysis and yields of C-0h, E-1h, and E-6h FPH made from each of the eight fish mince batches varying in infection level are shown in Figure 3.2a and Figure 3.2b, respectively. Increasing infection level up to ~ 10^7 *K. paniformis* spores per gram mince was accompanied by large increases in free amino group content of E-1h and E-6h FPH during

autolysis, with more moderate increases at infection levels beyond that (Figure 3.2a). These results are consistent with those reported by Samaranayaka and others (2006) and in Chapter 2 that a strong linear relationship ($r = 0.957$, $p = 0.000$) was observed between spore counts below 10×10^6 per gram fish mince and the endogenous proteolytic activity; beyond that infection level, proteolytic activity remained high but did not change further ($p > 0.05$) with the infection level up to 100×10^6 spores per gram fish mince. Nevertheless, the present study shows highest ($p < 0.05$) extent of autolysis in E-6h FPH that was prepared from fish mince Inf-100 with the highest infection level (Figure 3.2a).

Yields of FPH also increased with fish mince infection level, with lowest yields for C-0h FPH (Figure 3.2b). It was interesting to note that C-0h FPH yields were higher for fish mince batches Inf-30 and Inf-100 compared to other C-0h FPH from fish mince with lower infection levels, indicating possible autolysis of fish mince during storage or handling of samples prior to the actual hydrolysis process, due to high protease levels present in the heavily parasitized fish mince. For all fish mince batches, even though the free amino group contents were significantly higher for E-6h FPH than E-1h FPH (Figure 3.2a), there were only small differences in yields (Figure 3.2b). This may be due to proteolysis of peptides already present in the solution phase rather than of the less soluble intact fish muscle proteins during autolysis beyond 1 h. This was also confirmed by having comparatively similar total amino acid contents for E-1h (604 mg/g sample) and E-6h (555 mg/g sample) FPH made from Inf-30 fish mince (based on amino acid analysis; **Appendix I**).

3.3.3.2 ABTS radical scavenging capacity

Similar to the yields and extents of hydrolysis, control FPH (C-0h) possessed the lowest ABTS radical scavenging ability (Figure 3.2c). E-1h and E-6h FPH made with fish mince batches Inf-7, Inf-9, Inf-20, Inf-30, and Inf-100 could reduce more than 50 % of the ABTS radicals in the assay media at 66.67 µg/mL sample concentration. For these fish mince, little or no difference in ABTS radical scavenging activity resulted by autolyzing up to 6 h compared to 1 h autolysis, confirming that 1 h autolysis at 52 °C and pH 5.50 is sufficient in making antioxidative FPH from parasitized Pacific hake. The results from Section 3.3.2 also indicated that E-1h FPH possessed significantly ($p < 0.05$) higher DPPH radical scavenging ability compared to other FPH made through autolysis. However, it is important to note that

E-1h FPH displaying >50 % radical scavenging power at 66.67 µg/mL sample concentration in the ABTS assay was only obtained from fish mince infected by at least ~10^7 *K. paniformis* spores per g fish mince (Figure 3.2c).

3.3.3.3 Linoleic acid peroxidation system

A linoleic acid peroxidation system was also used to assess antioxidant activities of E-1h FPH made with fish mince batches having different *K. paniformis* infection levels. In this study, 12.0 ± 6.6 %, 55.0 ± 5.1 %, 81.9 ± 4.0 %, and 85.7 ± 1.8 % inhibition of lipid peroxidation in the system was observed after 3 days of storage with E-1h FPH (at 0.2 mg/mL assay concentration) made with Inf-0, Inf-2, Inf-30, and Inf-100 fish mince, respectively. Therefore, similar to the TEAC assay results (Figure 3.2c), inhibition of lipid peroxidation by E-1h FPH produced from Inf-0 and Inf-2 Pacific hake fish mince was significantly ($p< 0.05$) lower than E-1h FPH from Inf-30 and Inf-100 fish mince.

3.3.4 Comparison of E-1h FPH to commercial antioxidants

E-1h FPH made from Inf-30 fish mince was assessed further for its antioxidant potential in comparison with some commercial antioxidants. TEAC value of E-1h FPH evaluated using the ABTS radical cation decolorization method was 262 ± 2 µmol/g freeze dried sample, compared to 14.29 and 2.27 mmol/g for BHA and BHT, respectively. Total antioxidant capacity measured as ORAC value for E-1h FPH was 225 ± 17 µmol Trolox equivalents/g freeze dried sample. This indicates higher total antioxidant activity measured as Trolox equivalents for Pacific hake E-1h FPH compared to ORAC values reported for different fruits such as strawberries, blueberries and raspberries (approx. 150, 200 and 100 µmol Trolox equivalents/g dry matter, respectively) (Wang and Lin 2000; Prior and others 1998). These fruits contain phenolic compounds which have been reported to function as antioxidants in improving human health (Margetts and Buttriss 2003). Therefore, FPH such as E-1h made using Pacific hake could also potentially possess significant health benefits through the antioxidant function attributed to its constituent peptides and/or amino acids.

E-1h FPH inhibited lipid peroxidation in a linoleic acid model system by 73 % and 90.8 % at 42 and 162 hrs of incubation, respectively (Figure 3.3). It is interesting to note that the

antioxidant activity of E-1h FPH was comparable to that of BHA until 92 hrs of incubation, and higher ($p< 0.05$) than BHA when the incubation period was extended up to 162 hrs. Moreover, antioxidant potential of E-1h FPH in this linoleic acid/ethanol/water model system was higher ($p< 0.05$) than α-tocopherol, a well known lipid soluble natural antioxidant, during 92-162 days of storage at 40 °C. Inhibition of lipid peroxidation by BHT was, however, higher ($p< 0.05$) than that of E-1h FPH throughout the incubation period (Figure 3.3).

A reduction of antioxidant activity of BHA was observed at 162 h of storage, while antioxidant capacity of α-tocopherol also decreased over storage time (Figure 3.3). In contrast, comparatively high antioxidant activity was observed for Pacific hake E-1h FPH even at 162 h of storage. Therefore, E-1h FPH may have potential to be used as an antioxidant in oil-in-water emulsion type food systems to inhibit lipid peroxidation for a longer time period than that of BHA and α-tocopherol. Similar results were reported for DPPH radical scavenging capacity of a porcine myofibrillar protein hydrolysate which continued to act as an effective radical scavenger in a non-polar system even after 1 h of incubation with DPPH radicals, whereas the scavenging effect of α-tocopherol had reduced gradually over 1 h (Saiga and others 2003). Peptides from the hydrolysates of yellowfin sole (Jun and others 2004), Alaska Pollack (Je and others 2005b), conger eel (Ranathunga and others 2006), and capelin (Shahidi and Amarowicz 1996) also showed inhibitory activity in the linoleic acid model system. Shahidi and Amarowicz (1996) also reported the concentration dependency of antioxidative potential of harp seal protein hydrolysates in a β-carotene/linoleate model system, with a prooxidative effect at 2 mg/mL assay concentration, compared to a weak antioxidant effect ($p< 0.05$) at 0.2 or 0.4 mg/mL concentration after 45, 105-120, and 30-120 min incubation. Antioxidative activity of these protein hydrolysates could be due to the ability of peptides to interfere with the propagation cycle of lipid peroxidation, thereby slowing radical mediated linoleic acid oxidation.

3.4 Conclusions

The present study clearly demonstrates that fish protein hydrolysates with antioxidant properties may be prepared using parasitized Pacific hake fish fillet mince through one hour autolysis at 52 °C and pH 5.50. The infection level of fish mince should be ~10^7 *K. paniformis* spores per gram fish mince or higher in order to obtain E-1h FPH with high antioxidant potential through autolysis. Since Pacific hake are commonly infected with *K. paniformis* at these high levels, the muscle from these fish possesses sufficiently high endogenous proteolytic activity for autolytic production of antioxidative FPH. Alternatively, FPH with high antioxidant potential may be produced from Pacific hake without considering the *Kudoa* infection level by conducting one hour hydrolysis with addition of 2 % commercial enzymes such as Validase® BNP or Flavourzyme® 500L, after first inactivating the endogenous enzymes.

The potent antioxidant activity of E-1h FPH compared to BHA and α-tocopherol, particularly in inhibiting lipid peroxidation in a linoleic acid model system over prolonged storage, warrants further basic research to characterize the peptides that are responsible for these antioxidant properties, as well as applied research to investigate applications of the FPH as food and nutraceutical ingredients. This research is currently underway in our laboratory.

Table 3.1 Extent of hydrolysis and antioxidant properties of fish protein hydrolysates (FPH) prepared by no (C), endogenous (E), Validase® BNP only (V) or with endogenous (E+V), Flavourzyme® 500L only (F) or with endogenous (E+F) enzyme treatments using Inf-20 and Inf-30 fish mince[1]

FPH	Yield (%)	α-amino group content[2]		DPPH· scavenging capacity[3] (%)		$ABTS^{\cdot+}$ scavenging capacity[4] (%)	Reducing power[4] (abs at 700 nm)	Fe^{2+} ion chelation capacity (%)[5]
	Inf-20	**Inf-20**	**Inf-30**	**Inf-20**	**Inf-30**	**Inf-30**	**Inf-30**	**Inf-30**
C-0h	NA[6]	0.216^{a}	0.252^{a}	NA	NA	28.2^{a}	NA	NA
C-1h	NA	0.225^{a}	0.297^{a}	NA	37.9^{e}	35.3^{b}	0.439^{d}	42.9^{f}
C-2h	14.8	0.234^{a}	0.314^{a}	1.61^{a}	36.3^{e}	41.8^{c}	0.545^{f}	46.4^{f}
E-0h	19.7	0.320^{a}	0.337^{a}	NA	51.4^{g}	46.9^{d}	0.515^{ef}	18.1^{cd}
E-1h	47.7	0.960^{b}	0.964^{b}	73.3^{e}	61.3^{i}	56.6^{e}	0.486^{e}	7.65^{a}
E-2h	55.4	1.25^{c}	1.13^{c}	60.4^{d}	41.5^{f}	61.9^{g}	0.523^{ef}	15.0^{bc}
E-3h	51.0	1.50^{d}	1.44^{d}	59.3^{d}	33.0^{d}	62.0^{g}	0.399^{c}	18.0^{cd}
E-4h	48.2	1.36^{c}	1.41^{d}	41.0^{c}	23.7^{b}	57.6^{ef}	0.323^{b}	23.8^{e}
E-5h	50.2	NA	1.54^{d}	NA	22.5^{b}	58.4^{efg}	0.356^{b}	14.8^{bc}
E-6h	52.1	1.75^{e}	1.83^{e}	12.2^{b}	26.4^{c}	59.0^{efg}	0.353^{b}	6.65^{a}
E+V-1h	NA	2.71^{g}	1.86^{ef}	NA	15.8^{a}	61.1^{fg}	0.227^{a}	19.8^{d}
E+V-2h	64.2	3.25^{i}	2.38^{g}	18.8^{b}	16.6^{a}	59.2^{efg}	0.257^{a}	21.2^{de}
V-1h	NA	1.52^{d}	1.20^{c}	NA	57.0^{h}	57.2^{ef}	0.603^{g}	12.9^{b}
V-2h	41.5	1.91^{f}	1.98^{f}	58.1^{d}	27.0^{c}	45.1^{cd}	0.415^{cd}	19.2^{d}
E+F-2h	63.8	3.10^{h}		20.7^{b}				
F-2h	49.9	2.03^{f}		56.8^{d}				

[1]Average results from triplicate analysis. Values within a column bearing different superscript letters (a-i) are significantly different $P< 0.05$

[2]mmol L-Leucine equivalents/g fish protein, measured using TNBS method

[3]At 3 mg/mL assay concentration of FPH

[4]At 66.67 µg/mL assay concentration of FPH

[5]At 5 mg/mL assay concentration of FPH, [6]NA = Not analyzed

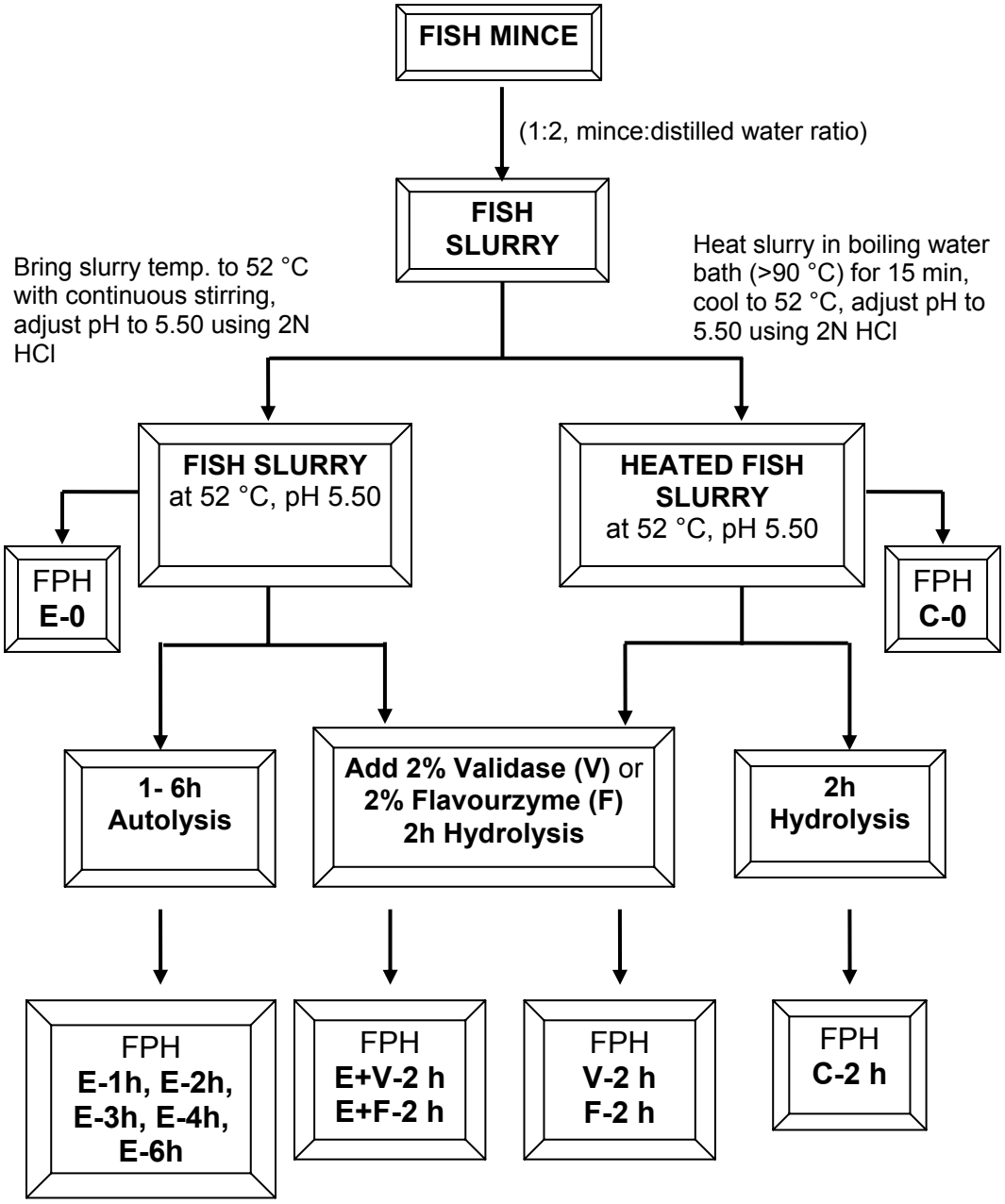

Figure 3.1 Process of fish protein hydrolysate (FPH) production with no (C), endogenous (E), Validase® BNP only (V) or with endogenous (E+V), Flavourzyme® 500L only (F) or with endogenous (E+F) enzyme treatments.

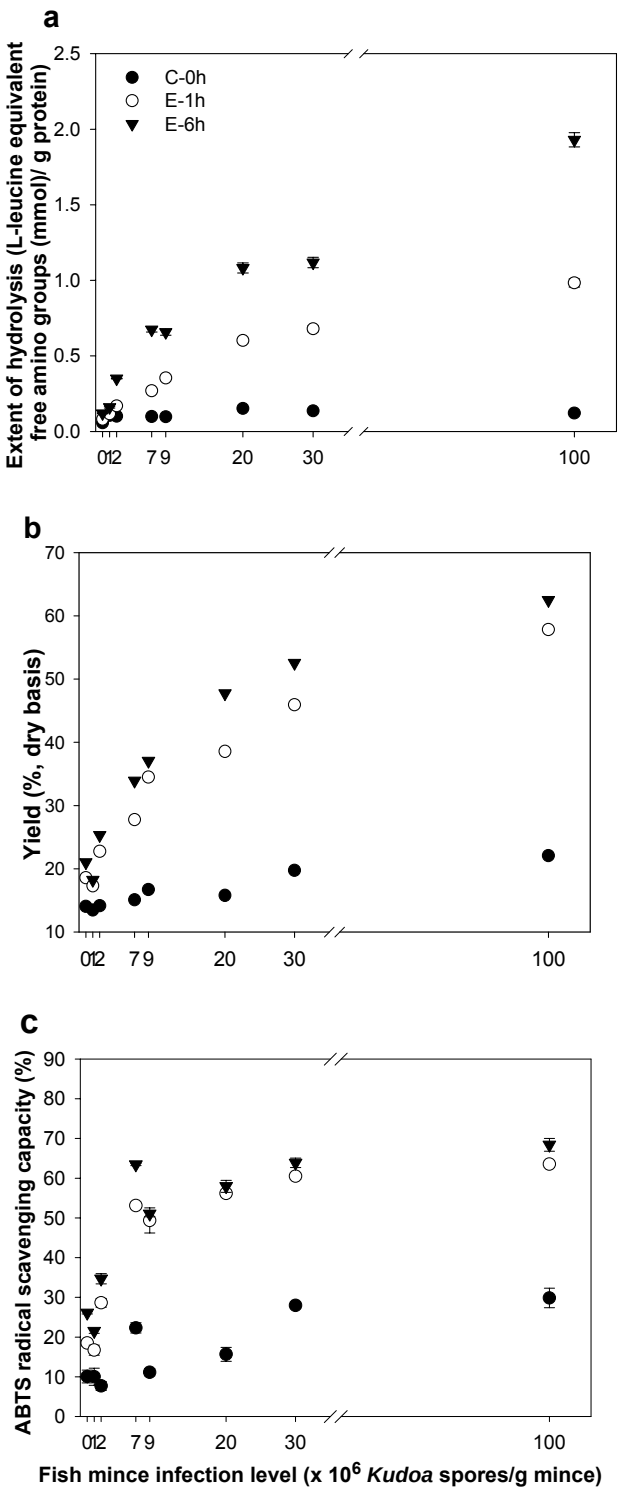

Figure 3.2 Extent of hydrolysis (a), yield (b), and ABTS radical scavenging capacity (at 66.67 µg/ml assay concentration) (c) of fish protein hydrolysates (FPH) made by autolysis of fish mince batches with different *K. paniformis* infection levels. Values and error bars in Figures 3.2(a) and (c) are the mean and standard deviation from triplicate analyses.

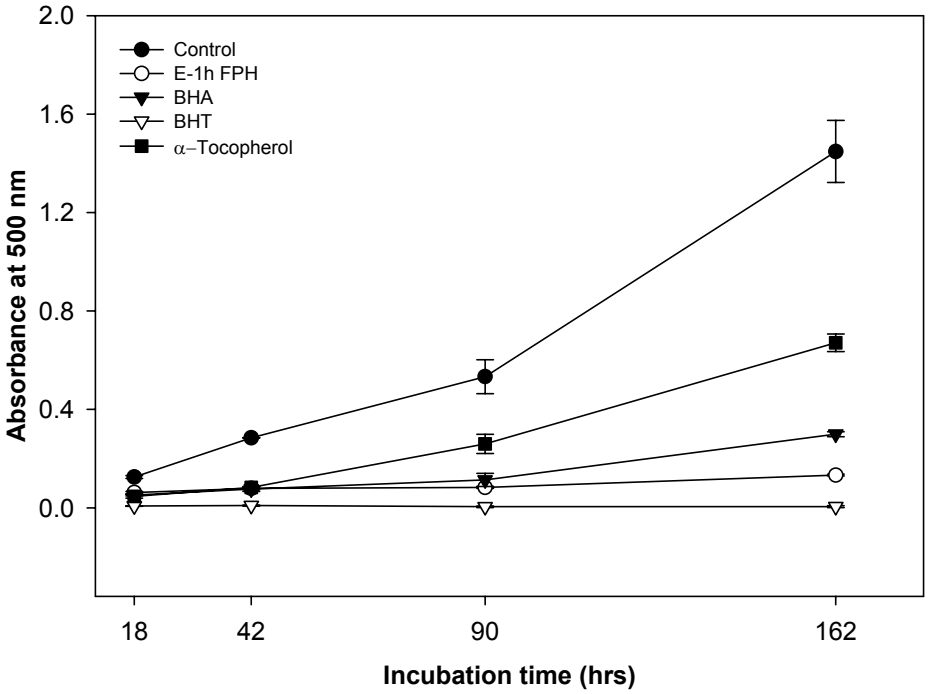

Figure 3.3 Inhibition of lipid peroxidation by E-1h fish protein hydrolysate (FPH), BHA, BHT, and α-tocopherol at 0.02 % sample concentration in a linoleic acid auto-oxidation system incubated at 40 °C for seven days. Values and error bars are the mean and standard deviation from triplicate analyses.

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CHAPTER 4 Antioxidative and Angiotensin-I-Converting Enzyme Inhibitory Potential of Pacific Hake (*Merluccius productus*) Fish Protein Hydrolysate Subjected to Simulated Gastro-intestinal Digestion and Caco-2 Cell Permeation[1]

4.1 Introduction

Protein hydrolysates derived from different food sources, including fish (Amarowicz and Shahidi 1997; Wu and others 2003; Jun and others 2004; Samaranayaka and Li-Chan 2008), soy (Chen and others 1995; Pena-Ramos and Xiong 2002), egg white (Tsuge and others 1991; Davalos and others 2004), and milk (Suetsuna and others 2000; Rival and others 2001; Kitts and Weiler 2003; Hernández-Ledesma and others 2005), have been reported to possess antioxidative properties. Recently, it has been demonstrated that protein hydrolysates exhibiting antioxidative potential may also contain peptides with other biological activities, such as angiotensin-I-converting enzyme (ACE)-inhibitory activity. Some examples of this include protein hydrolysates made from fermented milk (Hernández-Ledesma and others 2005), human milk and infant formula (Hernandez-Ledesma and others 2007), chum salmon cartilage (Nagai and others 2006), and egg albumen (Davalos and others 2004), which contained both antioxidative and (ACE) inhibitory peptides. A protein hydrolysate recovered from defatted *Brassica carinata* seed flour was shown to contain peptides with antioxidative, ACE-inhibitory, and hypocholesterolemic activity (Pedroche and others 2007). These protein hydrolysates with multiple bioactivities could be useful in formulating functional food products that target to reduce the symptoms of oxidative stress, hypertension and possibly dyslipidemia, all common to coronary heart disease.

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Numerous reports have also demonstrated different antioxidative capacities of peptides derived from a number of distinct marine species (Suetsuna 1999; Kim and others 2001; Rajapakse and others 2005; Mendis and others 2005; Thiansilakul and others 2007a,b; Kim and others 2007). Many of these studies have relied on the use of chemical based antioxidant tests that do not have the capacity to establish the true efficacy of antioxidative activity of potentially bioactive peptides that is influenced by intestinal cell permeability and interaction with intracellular sources of oxidative stress. Moreover, there is a little known about the potential for multiple bioactivity of protein hydrolysates derived from fish species, including Pacific hake (*Merluccius productus*) which is an under-valued fish caught off the waters of North West Pacific.

Effects of simulated gastrointestinal (GI) digestion, intestinal cell permeability, and interaction with intracellular sources of oxidative stress are three important parameters to be taken into consideration when determining the bioavailability and bioactivity of food-derived peptides. Even though animal studies and human clinical trials are the best way to assess the *in vivo* efficacy, i*n vitro* cultured cell model systems allow for rapid, inexpensive screening of the potential bioactive compounds for their bioavailability and metabolism. Human adenocarcinoma colon cancer (Caco-2) cell monolayers have been the most commonly reported in the literature for studying intestinal permeability of bioactive compounds due to their similarity to the intestinal endothelium cells (Vermeirssen and others 2005; Liu and Finley 2005). Cell culture models can also be used to evaluate cytotoxicity of bioactive compounds at concentrations to be used to exert the desired bioactivity in the body, as well as to study the potential to inhibit intracellular oxidation and to reduce inflammatory responses by antioxidative compounds (Elisia and Kitts 2008).

In the present study, Pacific hake FPH that had previously been identified as a potential source of antioxidative peptides based on *in vitro* chemical assays (Samaranayaka and Li-Chan 2008) was tested for its potential to also exhibit ACE-inhibitory capacity. Although some research has been conducted to study the effects of simulated GI digestion (Fujita and others 1995; Ruiz and others 2004; Lo and Li-Chan 2005; Lo and others 2006; Miguel and others 2006), potential bioavailability and bioactivity (Nakashima and others 2002; Satake and others 2002; Matsui and others 2002; Jung and others 2006; Vermeirssen and others 2005) of ACE-inhibitory peptides, there is less known about whether peptides contained in

these hydrolysates have antioxidative properties (Hernandez-Ledesma and others 2007; Pedroche and others 2007), and furthermore whether they are bioavailable. *In vitro* simulated GI digestion of Pacific hake FPH was therefore employed to study possible effects of digestion on antioxidative and ACE-inhibitory capacity. Further, the Caco-2 cell monolayer permeability model (Vermeirssen and others 2004) was used to investigate intestinal transport of Pacific hake digestion derived peptides and the potential bioactivity of those peptides that exhibited permeability.

4.2 Materials and Methods

4.2.1 Materials

Pepsin (P 7012, 3,300 units/mg solid activity), pancreatin (P 7545, activity equivalent to 8x U.S.P. specifications)**,** 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH), angiotensin-I-converting enzyme (ACE) from rabbit lung, hippuryl-histidyl-leucine tetrahydrate (HHL), 2,4,6-trinitrobenzenesulphonic acid (TNBS), trifluoroacetic acid (TFA), Hank's balanced salt solution (HBSS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON)**.** Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Invitrogen Canada (Burlington, ON). Caco-2 (ATCC accession number HTB-37) cells were obtained from the American Type Culture Collection (Manassas, VA).

4.2.2 Preparation of fish protein hydrolysate (FPH)

Fillets from 10 Pacific hake fish harvested off the coast of Vancouver Island, Canada (48.4^{o}N 125.2^{o}W) were used to prepare FPH. Whole fish (average weight 671 ± 89 g) were transported on ice to the University of British Columbia Food Science Laboratory within two days after capture, individually packed in polyethylene bags, labeled and stored frozen (-25

°C) until used. Fish were thawed in a cold room (4 °C) for 16 h, filleted, de-skinned, and then ground twice using a grinder (BEEM Gigant, Butcher & Packer Supply Company, Detroit, MI) with a 4 mm screen in order to make fish mince.

FPH was prepared by autolysis as previously reported (Samaranayaka and Li-Chan 2008). Briefly, a slurry of fish mince in distilled water (at a weight ratio of 1:2) was autolyzed by incubation at pH 5.50 and 52 °C for 1 h. After incubation, the slurry was heated in a boiling water bath (>90 °C) for 15 min, centrifuged at 17,000 x g for 20 min, and filtered through 2 layers of cheese cloth. The filtrates were adjusted to pH 7.0 and freeze dried. Freeze dried FPH samples were ground using a pestle and a mortar, then stored in sealed vials at -18 °C until used for further analysis.

4.2.3 Amino acid composition

FPH was analyzed for amino acid composition by the Advanced Protein Technology Center at The Hospital for Sick Children (Toronto, ON). Amino acid analysis was conducted on a Waters Pico-Tag HPLC system after precolumn derivatization of acid digested (i.e. 6 M HCl with 1 % phenol at 110 ^{o}C for 24 h) sample with phenylisothiocarbamate. Performic acid oxidation was performed before determining the Cys content, whereas methanesulfonic acid hydrolysis was used in determining the Trp content. The results were used to determine both the total content of amino acids in the sample as well as the relative amino acid composition, which was expressed as g/ 100g amino acids.

4.2.4 Ultrafiltration

A portion of FPH was fractionated by ultrafiltration (UF) using an Amicon® Model 8400 UF unit (Millipore Corporation, Billerica, MA) with Millipore membranes having molecular weight cut-offs of 10, 3 and 1 kDa. The fractions were collected as follows: >10 kDa, peptides retained without passing through 10 kDa membrane; 3-10 kDa, peptides permeating through the 10 kDa membrane but not the 3 kDa membrane; 1-3 kDa, peptides permeating through the 3 kDa membrane but not the 1 kDa membrane; <1 kDa, peptides permeating through the 1 kDa membrane. All fractions recovered were lyophilized and stored in sealed vials at -18 °C.

4.2.5 Simulated gastro-intestinal (GI) digestion

A modified method based on Hernández-Ledesma and others (2004) and Lo and Li-Chan (2005) was used for *in vitro* pepsin-pancreatin digestion of FPH and selected UF fractions. Sample (100 mg) was first dissolved in 10.0 mL distilled water in an Erlenmeyer flask and pH was adjusted to 2.0 with 5N HCl. Pepsin (enzyme to substrate ratio of 1:35 w/w) was added and the sealed flask was incubated in a shaking incubator for 1 h at 37 °C. The pH was then adjusted to 5.3 with a saturated $NaHCO_3$ solution and further to pH 7.5 with 5N NaOH. Pancreatin (enzyme to substrate ratio of 1:25 w/w) was added to the mixture, which was incubated again with shaking for 2 hrs at 37 °C, before being submerged in boiling water for 10 min to terminate the digestion. Aliquots of the digested sample (referred to as FPH-GI) were diluted to the appropriate concentrations of peptides for antioxidative capacity, ACE-inhibitory activity, and cell permeability assays, as well as for reversed-phase HPLC analysis, as described in the following sections.

4.2.6 Caco-2 cell permeability assay

Caco-2 cells were cultured in DMEM containing 10 % fetal bovine serum, penicillin (100 unit/mL), and streptomycin (100 μg/mL), in a fully humidified atmosphere with 5 % CO_2 at 37 °C. Cell culture medium was replaced every other day and cells were subcultured weekly. Following confluence, Caco-2 cells (0.3 mL of 2×10^5 cells/mL) were seeded on the cell culture insert (0.32 cm^2, 0.4μm pore size, BD Biosciences Canada, Mississauga, ON) with 0.8 mL medium in each well of the 24-well cell culture companion plate. Cell culture medium was carefully changed every other day for at least 21 days, until the Caco-2 cells were fully differentiated as monolayers. Monolayer integrity was monitored by measuring transepithelial electrical resistance (TEER) using a Millicell® Voltohmmeter (Millipore Corp., Bedford, MA, USA), both at the beginning and at the end of the assay. Cell viability measurements were also made before and after the experiment using the MTT assay (Mosmann 1983).

Cell permeability measurements were conducted in triplicate. Only monolayers with TEER of at least 900 Ω were used for the transport study. After washing cells twice with HBSS buffer, 0.15 mL of FPH or GI-digested sample (5 mg/mL in HBSS) was added to the

insert and 0.6 mL HBSS was added to the well. Normal culture conditions (i.e., pH 7.0 on both sides, in an incubator set at 37 °C) were maintained. After 2 h, permeates containing peptides passing through the cells into each receiving well containing 0.6 mL HBSS were collected and used directly for HPLC analysis according to the procedure described below. Peptide concentration analysis was based on amino group content measured by the TNBS method (Adler-Nissen 1979) as described in Chapter 2 (Section 2.2.7). The efficiency of peptide transport expressed as percent permeability for each peak was calculated by considering the area under each fraction of the HPLC chromatogram as follows (Cinq-Mars and others 2008):

% Permeability = 100 x [peptide detected in receiver side]/[initial peptide added to the insert]

Permeates were also used directly for the ABTS-radical cation decolorization assay, while an aliquot from each permeate was concentrated seven times by freeze-drying and then re-constituting in HPLC-grade water in order to perform the ACE-inhibitory assay described below.

4.2.7 HPLC Analysis

HPLC analysis of FPH, FPH-GI as well as the permeates from the Caco-2 cell permeability assay was performed using an Agilent 1100 HPLC system equipped with a Jupiter C12 Proteo 90Å column (250 x 4.6 mm, 4 μ, Phenomenex, Torrance, CA) set at 30 ^{o}C. The mobile phase consisted of 0.05 % TFA in water (A) and 0.05 % TFA in acetonitrile (B). A flow rate of 1 mL/min was used with the following gradient - 0 % B at 0 min, increasing to 25 % B at 25 min and 80 % B at 30 min, and returning to 0 % B at 38 min. Fifty microliters of 5 mg/mL sample was injected onto the column and peptide peaks were monitored at 214 nm by a diode array detector.

Seven fractions were collected from HPLC of the FPH and FPH-GI samples and the procedure was repeated 19 times to collect sufficient sample for subsequent antioxidative and ACE-inhibitory assays. Pooled fractions from the 19 HPLC runs were freeze-dried after removing acetonitrile under a stream of nitrogen, and reconstituted in HPLC-grade water. To

facilitate a comparison of relative antioxidative and ACE-inhibitory activities for the 7 peak fractions, appropriate dilutions were made for each fraction depending on the relative amount recovered. Calibration curves for the areas of each peak in the HPLC profile were obtained by injecting different aliquots (1, 2, 5, 10 and 10 μL) of the FPH and FPH-GI samples (at 5 mg/mL concentration) before permeation through the Caco-2 cells. These calibration curves were used to calculate the amount of peptides that permeated through Caco-2 cells.

4.2.8 Antioxidative assays

4.2.8.1 Chemical assays

The antioxidative activity of FPH, UF fractions, FPH-GI, and fractions obtained after HPLC separation of FPH, FPH-GI, and the Caco-2 cell permeates of FPH and FPH-GI, were measured by the ABTS-radical cation decolorization assay using the method described in Samaranayaka and Li-Chan (2008). The Trolox equivalent antioxidative capacity (TEAC), which is the concentration of sample giving the same % inhibition of absorbance at 734 nm of ABTS radical cation as 1 mM Trolox, was determined for FPH and FPH-GI samples by assessing % inhibition of the absorbance of ABTS radicals by peptide solutions at four different final assay concentrations (6.67, 16.67, 33.33, and 66.67 μg/mL) in comparison to the values for a standard curve prepared with Trolox (0-20 μM final assay concentrations). Since the Caco-2 cell permeates were analyzed at one concentration, results were expressed as percent ABTS radicals scavenged instead of TEAC values. UF fractions were also analyzed further using the oxygen radical absorbing capacity (ORAC) assay described by Kitts and Hu (2005) and Samaranayaka and Li-Chan (2008).

4.2.8.2 Intracellular antioxidative activity by Caco-2 cell assay

An intracellular antioxidant assay was performed on FPH using Caco-2 cells maintained in DMEM, supplemented with 10 % fetal bovine serum, penicillin (100 unit/mL), and streptomycin (100 μg/mL) following the procedure of Elisia and Kitts (2008). Cells were incubated at 37 °C in a fully humidified environment under 5 % CO_2, and Caco-2 cells

at passage 40–60 were used for the experiments. Cells were subcultured at 2–3 days interval before reaching 90 % confluency.

AAPH-initiated intracellular oxidation in cultured Caco-2 cells was performed using black 96-well plates. Serial dilutions of FPH (0.625–5 mg/mL) in 100 µL of HBSS were added to cells (0.3 mL of $3.2x10^5$ cells/mL) and incubated for 2 h at 37 °C. The FPH containing solution was removed and 100 µL of DCFH-DA probe (10 µM in HBSS) was added to the cells and incubated for 30 min. This was followed by the addition of 100 µL of AAPH (1 mM in HBSS) to the cultured cells after removing the probe. Fluorescence readings ($\lambda_{Excitation}$ = 485 nm, $\lambda_{Emission}$ = 527 nm) were recorded using a microplate reader (Fluoroskan Ascent FL, Labsystem, Helsinki, Finland) at 0, 1, and 2 h after adding AAPH. The positive control consisted of cells with the DCFH-DA probe and the AAPH peroxyl radical initiator added but in the absence of FPH. The negative control consisted of cells exposed to only the DCFH-DA probe. All results were expressed according to the formula:

$Fluorescence = Fluorescence_{ti} - Fluorescence_{t0}$

where $Fluorescence_{ti}$ = fluorescence reading taken at time point "ti" during measurement, and $Fluorescence_{t0}$ = initial fluorescence reading taken at the point when AAPH was added (time zero).

4.2.9 ACE-inhibitory activity assay

ACE inhibitory activity of FPH, FPH-GI, fractions obtained after HPLC separation of FPH and FPH-GI, and Caco-2 cell permeates of FPH and FHP-GI, were determined according to the method described by Cinq-Mars and Li-Chan (2007) with some modifications. Briefly, 30 μL of sample was first pre-incubated with 30 μL (2.5 mU) of ACE at 37 °C for 15 min before adding the ACE substrate HHL. The 15 min pre-incubation time was based on results of a preliminary study in which FPH sample at 0.286 mg/mL concentration was pre-incubated with ACE for 0, 10, 20, 30, and 60 min. After 15 min incubation, 150 μL of 7.8 mM HHL was added and the solution was further incubated at 37 °C for 1 h. All assay components were dissolved in 0.025 M sodium tetraborate buffer containing 0.3 M NaCl (pH 8.3) for a total assay volume of 210 μL. HCl (250 μL, 1 N) was used to terminate the reaction. To extract hippuric acid, 1.0 mL of ethyl acetate was added.

The solutions were then vortexed for 30 s and microcentrifuged at 2000 x*g* at room temperature for 5 min. After centrifugation, 0.7 mL of the ethyl acetate layer was removed into a 10 mm diameter clear glass tube and the solvent was evaporated by heating for 30 min at 120 °C (Analog Dry Block Heater, VWR International Ltd., Delta, BC, Canada). The remaining hippuric acid residue was redissolved with 1.3 mL of distilled water, and the absorbance (abs) was read at 228 nm in a quartz cuvette. The sample aliquot was replaced by buffer in the positive control, and both the sample and ACE aliquots were replaced by buffer in the negative control. The percent ACE inhibition was calculated as
{1–[(abs of sample – abs negative control)/(abs positive control – abs negative control)]}x100 %

IC_{50}, which is the concentration of peptides required to inhibit ACE activity by 50 %, was also calculated for FPH and FPH-GI by performing the assay at four different final assay concentrations between 0 and 0.3 mg/mL. The IC_{50} values could not be estimated for HPLC fractions since the actual assay concentration was not known, the fractions having been reconstituted based on average area under each fraction of HPLC chromatogram as described previously.

4.2.10 Cellular cytotoxicity

Cytotoxicity tests using the Caco-2 cell model system were performed in triplicate with FPH and FPH-GI at different concentrations (dissolved in 100 µL HBSS). Plates containing FPH and FPH-GI samples and cells were incubated for 24 h, followed by replacing medium with 100 µL of 0.5 mg/mL MTT. After further incubation for 4 h in the dark, 100 µL of 10 % SDS dissolved in 0.01 % HCl was added. The absorption at 540 nm was measured against reference wavelength at 690 nm in a microplate reader (ThermoLabsystems Multiscan Spectrum, Chantilly, VA). The control sample consisted of Caco-2 cells treated with 100 µL HBSS without FPH. Viability of treated cells was expressed as a percent of viable cells present in the control (Mosmann 1983).

4.2.11 Statistical analysis

All analyses were performed in triplicate. Statistical analyses were performed using the SPSS statistical program (SPSS 10.0, SPSS Inc., Chicago, IL). Tukey's test was used to evaluate differences among mean values for treatments at the $P<0.05$ significance level.

4.3 Results and Discussion

4.3.1 Antioxidative activity in ultrafiltration fractions from FPH

According to previous research conducted in our laboratory, FPH with high antioxidative potential could be prepared from Pacific hake through one-hour autolysis at 52 °C and pH 5.50 (Samaranayaka and Li-Chan 2008), using high level of cathepsin L-like endogenous proteases present in Pacific hake muscle (An and others 1994). The temperature and pH conditions used in the present study were the same as previously reported to be optimal for activity of the endogenous proteases (Samaranayaka and others 2006). *In vitro* chemical antioxidant assays revealed that the FPH contained activity in both polar, non-polar, as well as an oil-in-water emulsion system (Samaranayaka and Li-Chan 2008).

The results of the chemical based ABTS and ORAC assays on ultrafiltrated Pacific hake FPH fractions in the present study showed that peptides within the 1-3 kDa UF fraction recovered from FPH had the greatest antioxidative capacity (Table 4.1). These results are in agreement with former studies using other marine species that reported antioxidative peptides derived from FPH had molecular weights that ranged from 500 to 1800 Da (Wu and others 2003; Jun and others 2004; Kim and others 2007; Je and others 2005a,b; Ranathunga and others 2006).

4.3.2 Effect of simulated GI digestion on antioxidative potential of FPH

Typical peptide profiles obtained using reversed-phase HPLC separation of FPH and FPH-GI at similar concentration (20 µL injection of 5 mg/mL starting solution onto HPLC) are shown in Figure 4.1. The concentrations of HPLC chromatogram peaks 4, 5 and 6 were higher in GI-digested FPH than in undigested FPH, while the content of more hydrophobic

peptides (e.g. peak 7) decreased after GI digestion. This observation suggests that the increased appearance of Peaks 4-6 may have originated from products arising from the digestion of peptides present in peak 7.

ABTS radical scavenging capacity of the collected fractions separated from samples with and without GI-digestion both followed the same trend, with fraction 4 having the highest radical scavenging capacity followed by fraction 6 (Table 4.2). There was a significantly higher ABTS radical scavenging capacity for peptides present in peak 3 after GI digestion. Pepsin and pancreatin catalyzed hydrolysis of FPH during the simulated GI digestion could lead to modifications of the antioxidative peptide sequences already present in FPH, increased formation of existing antioxidative peptides, and/or the creation of new antioxidative peptides from sequences not previously exhibiting antioxidative capacity in FPH. The reason for the observed increase in antioxidative potential of FPH-GI is not certain, but could be due to one or a combination of the above mentioned possibilities. Since there was an increase in the number of peptides present in peaks 4 and 6 upon GI digestion, and without new major peaks appearing in the chromatogram of FPH-GI, we conclude that the pepsin-pancreatin digestion likely increased the abundance of antioxidative peptides already present in the FPH. Sannaveerappa and others (2007) reported that enzymatic breakdown of proteins present in an aqueous extract of herring under GI-like conditions could increase peroxyl radical scavenging activity and the potential to inhibit model LDL oxidation**.**

4.3.3 ACE-inhibitory activity

The FPH was analyzed further to assess its capability to act as an ACE-inhibitor *in vitro*. It is not uncommon for protein hydrolysates, or peptides recovered from them, to possess both antioxidative and ACE-inhibitory activity (Hernandez-Ledesma and others 2007) The results showed that Pacific hake FPH had ACE-inhibitory activity, with IC_{50} value of 235 µg solids/mL (Table 4.1), or 162 µg peptides/mL when expressed on the basis of 69.16 % amino acid content of FPH determined by amino acid analysis. Analysis of the UF fractions of FPH indicated that ACE-inhibitory activity was concentrated with peptides that were present in the 1-3 kDa UF fraction, having a IC_{50} value of 188 µg solids/mL (Table 4.1). A similar IC_{50} value of 165 µg peptides/mL was reported previously for an FPH

prepared using the same stock of Pacific hake captured near Vancouver Island, BC, but following a pre-incubation with an exogenous enzyme preparation (Protamex^{TM}) at pH 6.5 and 40 °C for 125 min (Cinq-Mars and Li-Chan 2007). It is of interest that ACE-inhibitory activity of non GI digested Pacific hake FPH in this study is similar to the ACE-inhibitory activity reported for pepsin-pancreatin digested human milk peptide fractions (e.g. IC_{50} of 240 µg/mL protein, Hernandez-Ledesma and others 2007).

The fact that ACE is a dipeptidase enzyme, makes it plausible for further hydrolysis reactions to occur which could thereby affect the activity of ACE-inhibitory peptides *in vivo*. Depending on the outcome of GI digestion or other enzyme action *in vivo*, bioactive peptides can be classified as either true inhibitor (i.e. ACE-inhibitory activity remain unchanged), pro-drug (i.e. increased ACE-inhibitory activity) or substrate (i.e. decreased ACE-inhibitory activity) types of ACE inhibitors (Fujita and others 2000). Pre-incubation of FPH with ACE indicated that the ACE-inhibitory activity decreased with pre-incubation time when tested at 10 min, and remained unchanged thereafter up to 60 min of pre-incubation at 37 °C (Figure 4.2). Therefore, the FPH produced in this study was categorized as being a "substrate" type inhibitor with respect to the action of ACE. Since the ACE-inhibitory activity remains unchanged after 10 min, samples were pre-incubated with ACE for 15 min before adding HHL substrate when performing the ACE assay during this study.

4.3.4 Effect of simulated GI digestion on ACE-inhibitory activity

ACE-inhibitory potential of FPH did not significantly change upon simulated GI digestion (Table 4.1). However, the effect of pepsin-pancreatin digestion was more pronounced for the partially purified ACE-inhibitory peptides present in the 1-3 kDa UF fraction where there was a significant decrease in ACE-inhibitory activity (Table 4.1). Out of the seven fractions obtained by separating FPH using HPLC, the highest ACE-inhibitory potential was measured in peak 1 (Table 4.2). This finding indicates that peptides derived from digested hake FPH with potential blood pressure lowering capability are in fact different from peptides present in FPH that exhibited antioxidative activity. From the characteristic retention times separating the different fractions, it can be concluded that peptides contained in peak 1, eluting earlier and showing higher ACE-inhibitory activity, are composed of relatively fewer hydrophobic amino acid residues and/or are of shorter chain

length and therefore more polar in nature due to the presence of charged amino and carboxyl terminal groups. In comparison, peptides exhibiting the most potent ABTS-radical scavenging capacity appear in peaks 4 and 6, and would in contrast contain more hydrophobic residues and/or a longer chain length. Moreover, a decrease in ACE-inhibitory activity was observed for peak 1 after *in vitro* GI digestion of the unfractionated FPH (Table 4.2). Further hydrolysis of ACE-inhibitory peptides into smaller peptides or free amino acids during GI digestion can result in a decreased relative ACE-inhibitory potential. During the present study, the ACE-inhibitory peptides originally present in whole FPH that were concentrated into 1-3 kDa UF fraction (Table 4.1) and to the peak 1 (Table 4.2) during RP-HPLC separation might have been broken down into less active peptide fragments or amino acids upon pepsin-pancreatin digestion. Since the ACE-inhibitory activity is measured on the basis of total material in each fraction, another possibility for the decrease in activity of peak 1 after GI digestion can be the formation of new peptides without significant ACE-inhibitory activity but eluting in this retention time range (Figure 4.1), which could dilute the active peptides in this fraction. Further, the ACE-inhibitory activity of peaks 4-6 did not significantly change upon GI digestion (Table 4.2) but the material in each peak greatly increased. It could therefore be suggested that new or more of the existing ACE-inhibitory peptides eluting in these retention time ranges might have formed during pepsin-pancreatin digestion. This could be the reason for not showing a significant change of the ACE-inhibitory activity of the unfractionated FPH upon GI digestion. Miguel and others (2006) reported that the ACE-inhibitory activity of two ovalbumin-derived peptides of YAEERYPIL and RADHPFL decreased upon *in vitro* simulated GI digestion even though the peptides were capable of exerting antihypertensive activity in spontaneously hypertensive rats (SHR). In contrast, several other studies have observed an increase in ACE-inhibitory activity upon GI-digestion. Two oligopeptides (IWHHT and IVGRPRHQG) isolated from a thermolysin digest of dried bonito exhibited enhanced activity following incubation with chymotrypsin and trypsin, respectively (Fujita and others 2000). Similarly, IC_{50} value of Pacific hake FPH prepared by hydrolyzing with Protamex was reduced to 90 µg peptides/mL upon simulated GI-digestion, compared to IC_{50} value of 165 µg peptides/mL for the undigested FPH (Cinq-Mars and others 2008). Studies by Wu and Ding (2002) using soy protein hydrolysates reported a 10 percent increase in IC_{50} value following *in vitro* GI-digestion of the <10 kDa

UF fraction. The ACE-inhibitory activity of protein hydrolysates made from fermented milk (Hernandez-Ledesma and others 2004) however, did not significantly change with *in vitro* pepsin pancreatin digestion. Therefore, given the diversity of outcomes reported for various hydrolysates and peptides, investigating the fate of bioactive peptides or precursor proteins and oligopeptides present in food during GI digestion and physiological uptake is an important step in screening for potential bioactive peptide sources.

4.3.5 Caco-2 cell permeate analysis and bioactivity measurements

Analysis of cellular permeability of the FPH peptides was performed only on HPLC peaks 2-6 since the broad and complex nature of HPLC peaks 1 and 7 (Figure 4.1) made it difficult to reliably estimate the percentage permeability for those two peaks. The results demonstrated that certain components present in peaks 2-6 from FPH and FPH-GI could permeate the Caco-2 cell monolayer (Table 4.3). Quantification of peptide concentration in permeates using the TNBS assay indicated that 5.04 % of FPH and 2.80 % of GI-digested FPH (out of the total amount of peptides loaded on the apical side of the Caco-2 cells) had passed through and were detected in the basolateral side of the cells. Although the permeability values expressed as percentage were lower for HPLC fractions of FPH-GI compared to FPH (Table 4.3), the total amounts transported for these components were actually higher in the permeates of FPH-GI than FPH, since the total content of these peaks was higher in FPH-GI than FPH. This finding suggests that transport of peptides in the FPH-GI sample across the Caco-2 monolayer may have reached a saturation point.

In the present study, peptides from FPH and FPH-GI that were transported through Caco-2 cells possessed ABTS-radical scavenging activities of 5.78 ± 0.43 % and 11.68 ± 1.01 %, respectively, when the assays were conducted using 65 µL of the undiluted permeates. Therefore, permeated peptides from FPH-GI indicated higher antioxidative activity compared to the peptides permeated from the undigested FPH. Both FPH and FPH-GI inhibited the intracellular oxidation of Caco-2 cells induced by AAPH substrate, with no significant difference between the two concentrations tested (0.625 and 1.25 mg/mL) (Figure 4.3). Since whole hydrolysate, instead of purified peptides, was used in these experiments to assess antioxidant activity of cell permeates and inhibition of intracellular oxidation, it is

difficult to state whether the antioxidative peptides present in FPH could penetrate the intestinal cell membrane in intact form or after modification by brush-border peptidases. Nevertheless, since HPLC of FPH and FPH-GI showed relatively high permeability of several fractions (Table 4.3), including peaks 4 and 6, which exhibited antioxidative potential (Table 4.2), it can be speculated that these peptides permeated through the Caco-2 cell monolayer and effectively contributed to the measured antioxidative activity exhibited by the permeates.

Only a few studies at the present time have attempted to assess the antioxidative potential of peptide hydrolysates or isolated peptides using cell culture, animal models or human clinical trials. Rajapakse and others (2005) reported free radical scavenging activity of a peptide derived from fermented mussel sauce (e.g. HFGBPFH) in cultured human lung fibroblast cells. Antioxidative activity was concentration-dependent up to 75 μg/mL, with no further protection on cell survival obtained at higher peptide concentrations. Seacure®, a commercially available fermented fish product made by controlled yeast fermentation of Pacific hake, claims to be beneficial for a variety of gut conditions. Fitzgerald and others (2005) studied the efficacy of this product using various models of epithelial injury and repair. When cultured rat epithelia and human colon cells were given the Seacure®, cell growth was significantly increased (at 1 mg/ml concentration, $p<0.01$) and the cell injury was significantly reduced (at 25 mg/ml concentration, $p<0.05$). The effects were attributed to the action of ethanol soluble di- and tri-peptides containing glutamine. A recent pilot human clinical trial using Seacure® has also shown a reduction in the degree of small intestinal damage caused by the non-steroidal anti-inflammatory drug, indomethacin (Marchbank and others 2008). This study also suggested that glutamine present in FPH might have contributed to the observed antioxidant activity via stimulation of intercellular glutathione production. About 19.2 % of the total amino acids present in FPH prepared using Pacific hake during the present study were also composed of glutamine and/or glutamic acid (Table 4.4), and therefore this compositional characteristic may be noteworthy in explaining further the *in vivo* potential for antioxidative functionality.

In the present study, although peptides that permeated through Caco-2 cells exhibited some degree of antioxidative activity, there was no similar measurable ACE-inhibitory activity of the permeates, even after concentrating by ~7 fold. We suspect that the

concentration of ACE-inhibitory peptides in permeates was too low to detect using our *in vitro* assay procedure. Alternatively, the ACE-inhibitory peptides may not have passed through Caco-2 cells, due either to physical limitation or moreover, to the possibility that they were degraded to non-active fragments by brush border peptidases. Our results (Table 4.2) show that most of the ACE-inhibitory peptides were concentrated into peak 1; nevertheless results of HPLC analysis of Caco-2 cell permeate for both FPH and FPH-GI indicated that there was no detectable recovery of peak 1 peptides which penetrated Caco-2 cells (data not shown). Our results are similar to findings reported by Vermeirssen and others (2005), where little or no ACE-inhibitory activity was observed for GI-digested whey and pea proteins following transepithelial procedures using Caco-2 cell monolayer, even though the peptides were found to be resistant to brush border peptidases. The pea digest did, however, exert a significant blood pressure lowering effect when injected at 50 mg protein per kg body weight into the femoral vein of SHR (Vermeirssen and others 2005). Qian and others (2007) also reported that a peptide, characterized as WPEAAELMMEVDP, and isolated from tuna dark muscle exhibited antihypertensive effect after oral administration to spontaneously hypertensive rats. Miguel and others (2008) reported an egg-derived peptide FRADHPFL was absorbed through the Caco-2 cell epithelium, even though it was also susceptible to brush border aminopeptidases which produced smaller peptide fragments prior to transport. A tripeptide, YPI, shown to be resistant to cellular peptidases was transported through the Caco-2 cell monolayer (Miguel and others 2008), as was an ACE-inhibitory tripeptide, VPP, that was transported across the Caco-2 monolayer at 2 % concentration (Satake and others 2002). From these studies, it may be concluded that three different transport routes; notably paracellular, fluid phase, and adsorptive transcytosis are involved in oligopeptide transport across the intestinal epithelium. Further, the contribution of each route and the affinity of individual peptides to transport across the membrane will depend upon the relative molecular size, hydrophobicity, as well as resistance to brush-border peptidases (Shimizu and others 1997; Satake and others 2002).

4.3.6 Cellular cytotoxicity

Viability of Caco-2 cells after treating with FPH and FPH-GI was assessed using the MTT assay which measures the ability of functional mitochondria to catalyze the reduction

of MTT to a formazan salt by mitochondrial dehydrogenases (Mosmann 1983). This assay was performed to assess possible toxic effects of FPH and FPH-GI to human cell systems, which would affect the interpretation of results from both the intracellular antioxidant and cell permeability assays. Cell viability ranged from 80-100 % when cells were incubated for 24 h with 0.625 to 5 mg/mL FPH or FPH-GI (Figure 4.4). It is interesting to note that no effect on cell viability was observed at the lowest concentration of 0.625 mg/mL (Figure 4.4), while this concentration was sufficient to significantly inhibit oxidation of Caco-2 cells after 2 h incubation at 37 °C (Figure 4.3). Further, Pacific hake FPH showed no toxicity to human hepatocellular liver carcinoma (HepG-2) cells when exposed at sample concentrations up to 1 mg/mL (**Appendix II**). The antioxidative peptide VKAGFAWTANQQLS (1519 Da), isolated from tuna backbone protein hydrolysate, was also reported to have no cytotoxic effect when tested on human lung fibroblast (MRC-5) and human endothelial (ECV304) cells (Je and others 2007).

4.4 Conclusions

Antioxidative FPH prepared from Pacific hake through autolysis was characterized by *in vitro* assay to be a 'substrate' type inhibitor of ACE. Antioxidative capacity of FPH increased after simulated GI digestion. Caco-2 cell permeation study and intracellular antioxidant assay under the experimental conditions used herein, showed good potential of FPH to act as an antioxidative agent, *in vivo*, at concentrations that were not toxic to the cells. Further work is underway using RP-HPLC mass spectrometry to determine the sequences of peptides in the fractions with antioxidative capacity, and further to evaluate the mechanism of their antioxidative capacity. The ACE-inhibitory potential of FPH remained unchanged upon simulated GI digestion but the Caco-2 cell permeate did not possess any ACE-inhibitory activity. Miguel and others (2006) suggested that inhibiting ACE activity is not the only mechanism by which peptides can act as antihypertensive agents. Even though the ACE-inhibitory activity of peptides may decrease upon the digestion by pepsin and pancreatic enzymes, or by brush border peptidases, the resultant peptide fragments might have the possibility to still contribute to hypotensive action by stimulating opioid receptors (Sipola and others 2002), acting on smooth muscles (Kuono and others 2005), or acting as

antioxidants to reduce oxidative damages that are mediators in cardiovascular pathologies (Touyz 2004). Future studies using *in vivo* animal model systems and human clinical trials, are therefore necessary to ascertain the required standards of evidence for both antioxidative and ACE-inhibitory potential of bioactive peptides derived from hake FPH before consumer use is recommended.

Table 4.1 Antioxidative and ACE-inhibitory capacity of Pacific hake fish protein hydrolysate (FPH), ultrafiltration fractions, and *in vitro* gastro-intestinal (GI) digested samples[1]

Sample	TEAC value[2]	ORAC value[2]	% inhibition of ACE [IC_{50}, µg/ml][3]
FPH	262 ± 2[a]	225 ± 17[a]	55.06 ± 0.66[c] [235]
>10 kDa	251 ± 4[a]	249 ± 10[a]	18.05 ± 3.88[a] [906]
3-10 kDa	305 ± 4[b]	255 ± 21[a]	43.40 ± 6.70[b] [329]
1-3 kDa	312 ± 1[b]	330 ± 18[b]	66.91 ± 4.38[d] [188]
<1 kDa	240 ± 10[a]	253 ± 17[a]	55.92 ± 3.30[c] [218]
GI digested			
FPH-GI	376 ± 3[c]	ND[4]	49.40 ± 2.10[bc]
1-3 kDa-GI	434 ± 17[d]	ND	46.55 ± 2.19[bc]

[1]Average results ± standard deviation from two replicate experiments analyzed in triplicate. Values within a column bearing different letters (a-d) are significantly different at $P< 0.05$.

[2]Trolox equivalent antioxidant capacity (TEAC) and Oxygen radical absorbing capacity (ORAC) values expressed as µmol Trolox equivalents/g sample.

[3]% inhibition of ACE was measured at 0.286 mg/mL sample concentration. IC_{50} values are shown within brackets.

[4]Not determined.

Table 4.2 Antioxidative and ACE-inhibitory capacities of HPLC fractions obtained from Pacific hake FPH and *in vitro* gastro-intestinal (GI) digested FPH[1]

HPLC peak # from Figure 4.1	Retention time (min)	% $ABTS^{\cdot+}$ scavenged		% Inhibition of ACE	
		FPH	FPH-GI	FPH	FPH-GI
1	2.5-3.5	2.73 ± 0.12[a]	2.73 ± 0.12[a]	83.33 ± 1.56[a]	48.19 ± 5.00[b]
2	6-7.4	2.81 ± 0.00[a]	2.90 ± 0.12[a]	25.42 ± 1.56[a]	7.96 ± 7.50[b]
3	8.5-9.5	50.04 ± 0.24[a]	70.67 ± 0.48[b]	40.45 ± 3.44[a]	38.90 ± 5.63[a]
4	9.51-10.3	98.21 ± 0.12[a]	97.95 ± 0.24[a]	38.02 ± 1.25[a]	28.29 ± 3.75[a]
5	13-14.2	45.27 ± 0.24[a]	43.39 ± 0.48[b]	11.05 ± 3.13[a]	13.04 ± 7.19[a]
6	18-19	81.84 ± 0.36[a]	83.55 ± 0.84[a]	11.27 ± 0.31[a]	19.89 ± 10.00[a]
7	29-37	10.32 ± 0.48[a]	6.05 ± 0.24[b]	NA[2]	NA

[1]Values are mean ± standard deviation from triplicate analysis. Values within each property (antioxidant or ACE-inhibitory activity) in a row bearing different letters are significantly different at $P<0.05$.

[2]No inhibitory activity.

Table 4.3 Caco-2 cell permeability of peptides from Pacific hake fish protein hydrolysate (FPH) and *in vitro* gastro-intestinal (GI) digested FPH

HPLC peak # from Figure 4.1	% permeation through Caco-2 cells[1]	
	FPH	FPH-GI
2	24.2 ± 0.8[a]	20.3 ± 0.6[b]
3	10.6 ± 0.3[a]	8.4 ± 0.6[a]
4	13.2 ± 1.6[a]	5.3 ± 0.6[b]
5	14.8 ± 0.9[a]	3.1 ± 0.2[b]
6	16.5 ± 0.3[a]	8.9 ± 0.8[b]

[1]Permeation (%) was calculated from the area of each peak in the HPLC chromatogram of FPH or FPH-GI analyzed before permeation, and in the permeates after apical-to-basolateral permeation. Values shown are the mean ± standard deviation of three replicates. Values in a row bearing different letters are significantly different at $P<0.05$.

Table 4.4 Amino acid composition of Pacific hake fish protein hydrolysate

Amino acid	Composition (g/100 g)[1]
Aspartic acid + Asparagine	10.33
Glutamic acid + Glutamine	19.04
Serine	4.00
Glycine	4.52
Histidine	1.91
Arginine	7.97
Threonine	4.03
Alanine	6.26
Proline	3.41
Tyrosine	2.84
Valine	4.78
Methionine	3.12
Isoleucine	3.67
Leucine	9.82
Phenylalanine	2.95
Lysine	10.35
Cysteine	0.88
Tryptophan	0.15

[1]Expressed as g/100 g amino acids. The total amino acid content expressed as a percentage of FPH dry matter was 69.16 %.

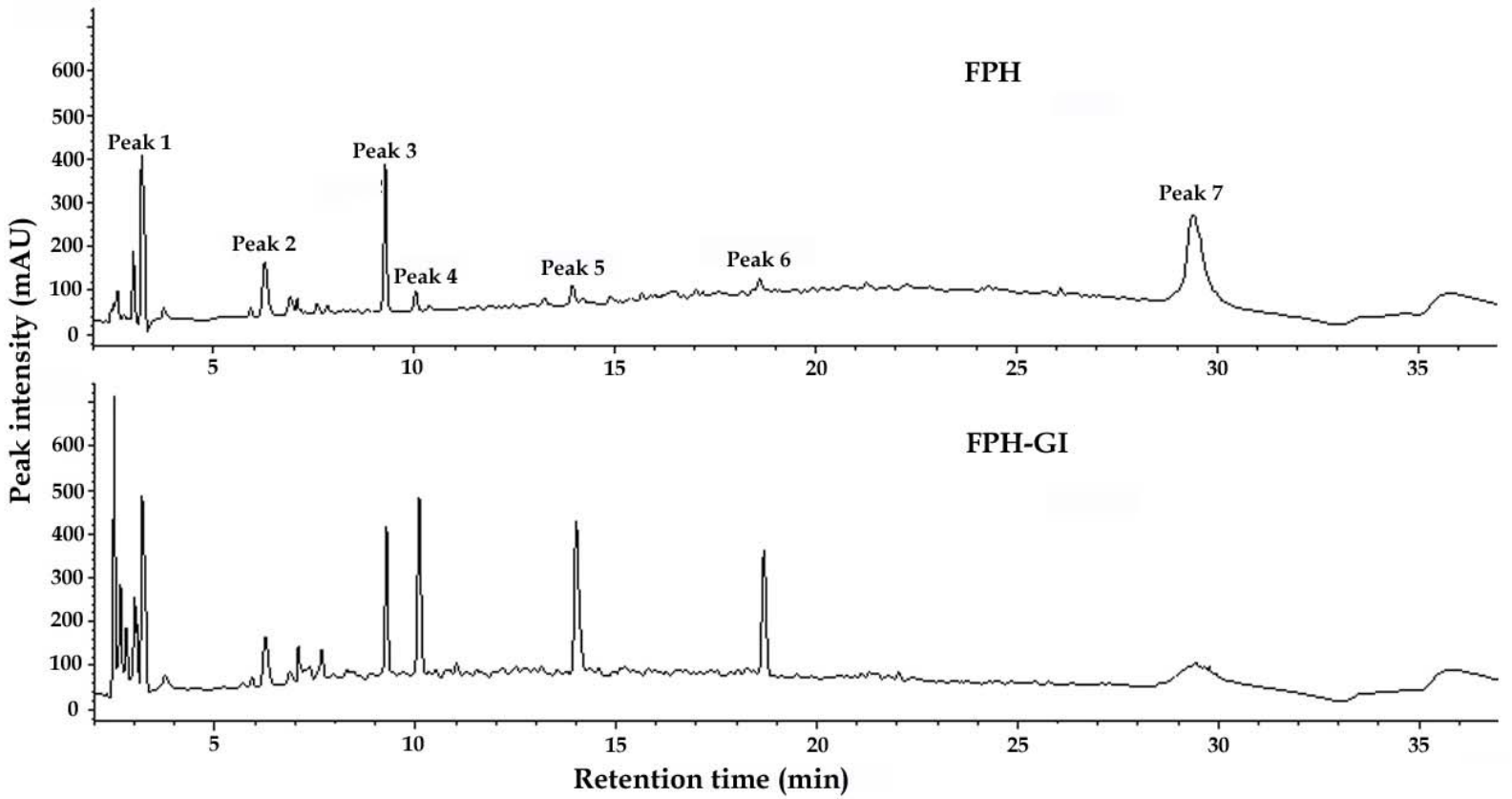

Figure 4.1 HPLC profiles of Pacific hake fish protein hydrolysate (FPH) and *in vitro* gastro-intestinal (GI) digested FPH (FPH-GI) at similar sample concentration (20 µL injection of 5 mg/mL solution) Retention time ranges for the HPLC fractions collected (i.e. peaks 1 to 7) are 2.5-3.5, 6.0-7.4, 8.5-9.5, 9.51-10.3, 13-14.2, 18-19, and 29-37 min.

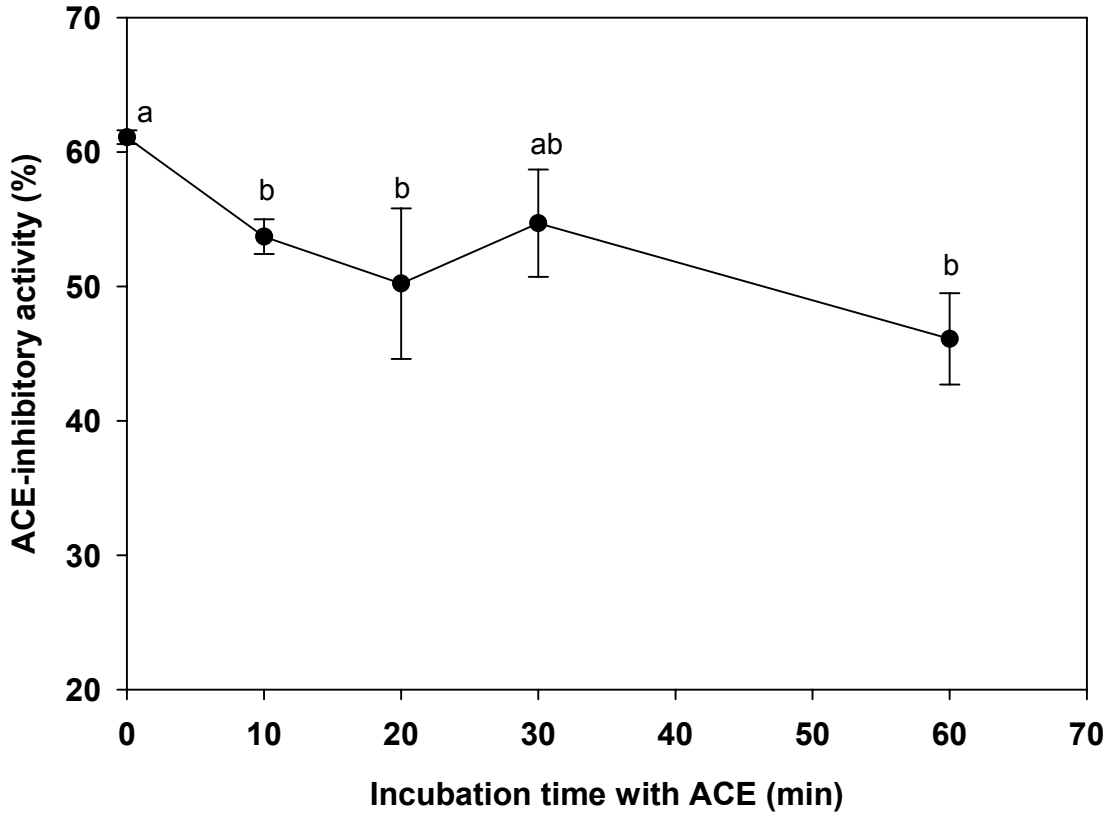

Figure 4.2 Effect of pre-incubation with ACE on ACE-inhibitory activity of Pacific hake FPH at 0.286 mg solids/mL assay concentration

Values shown are the mean ± standard deviation of three replicates. Data points bearing different letters are significantly different in ACE-inhibitory activity at $P < 0.05$.

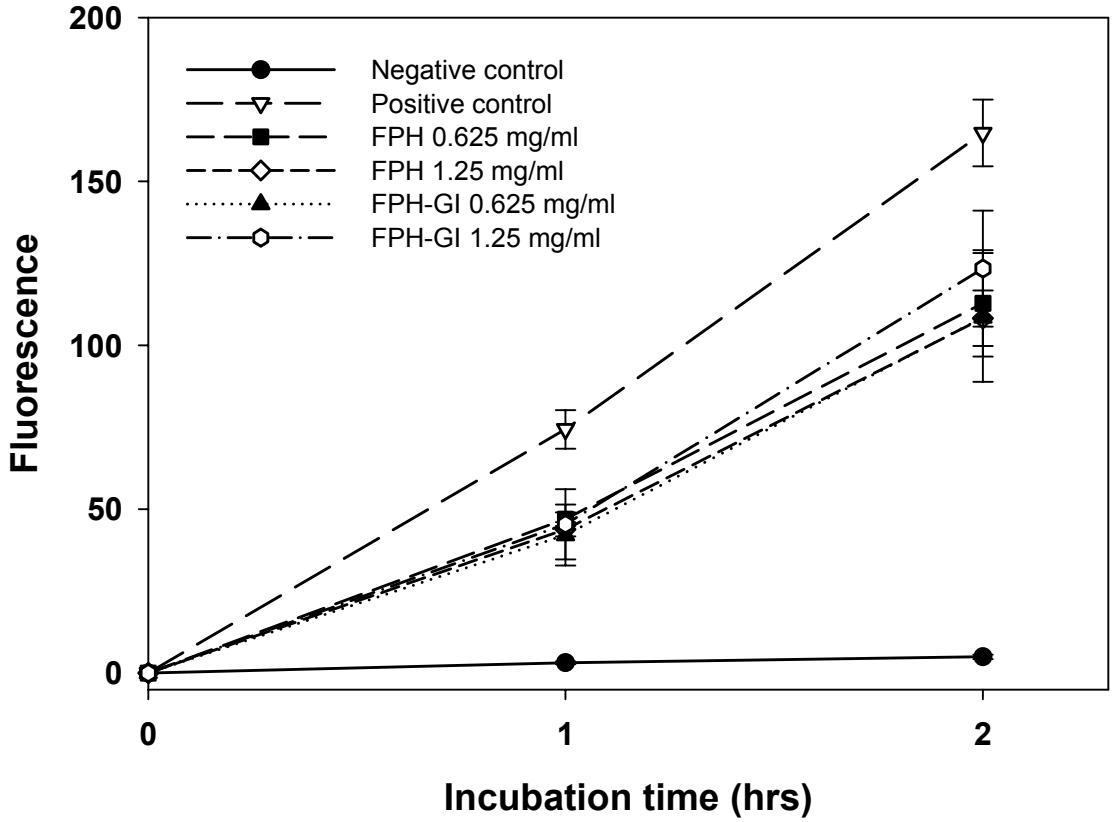

Figure 4.3 Inhibition of the 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH)-induced intracellular oxidation of Caco-2 cells by Pacific hake FPH and FPH-GI at 0.625 and 1.25 mg/mL sample concentrations

Values shown are the mean ± standard deviation of three replicates.

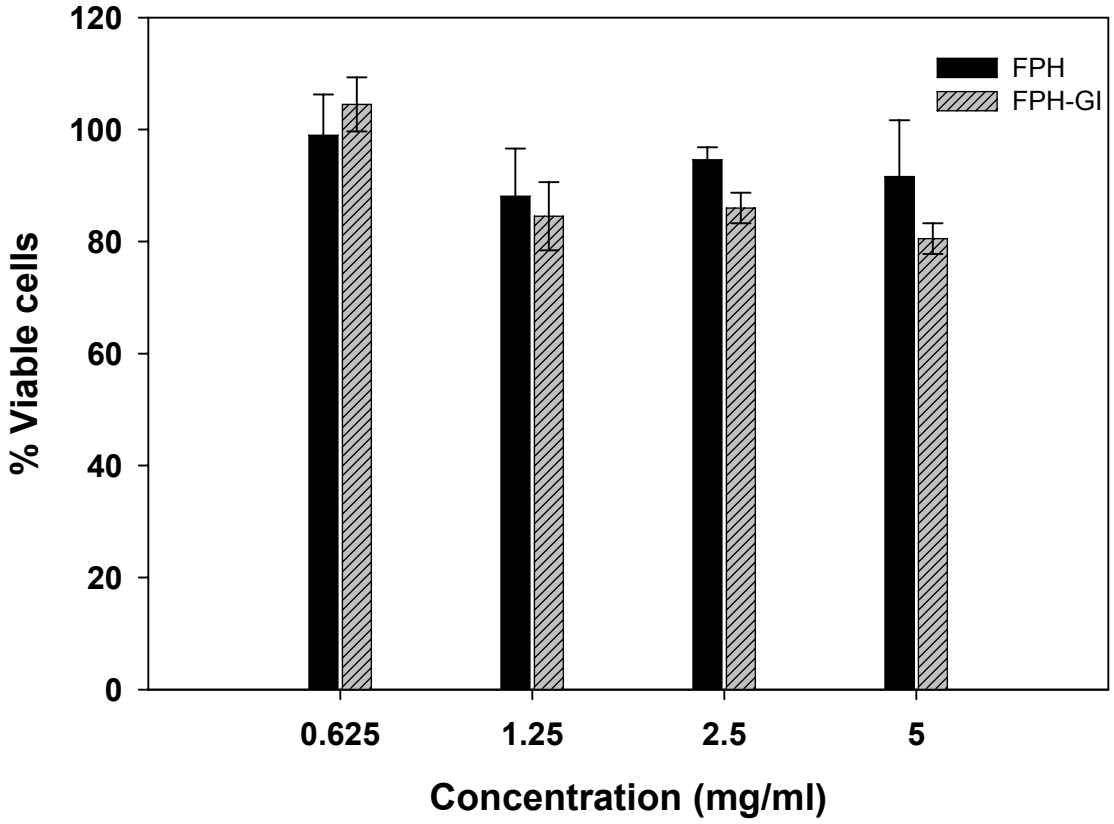

Figure 4.4 Viability of Caco-2 cells after incubation with Pacific hake FPH and FPH-GI at different concentrations

Values shown are the mean ± standard deviation of three replicates.

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CHAPTER 5 Identification of Antioxidative Peptides Present in a Fish Protein Hydrolysate Produced by an Autolysis of Pacific Hake (*Merluccius productus*)[1]

5.1 Introduction

Fish processing by-products, discards, as well as undervalued fish are a valuable source of raw material for the production of bioactive peptides from the protein components (Guérard 2007). Many different biological activities have been reported for fish protein hydrolysates (FPH) and specific peptide sequences derived from these hydrolysates (Shahidi 2005; Kim and Mendis 2006; Guérard 2007). In particular, antioxidative, antihypertensive, immunomodulatory, neuroactive, antimicrobial, mineral and hormonal regulating properties have been reported for many bioactive peptides (Bernet and others 2000; Jun and others 2004; Je and others 2005b; Duarte and others 2006; Jung and others 2006; Je and others 2007; Murray and FitzGerald 2007; Liu and others 2008).

Pacific hake (*Merluccius productus*), an undervalued fish which is harvested near Vancouver Island, BC, Canada, was successfully utilized for the production of antioxidative peptides with potential to be used as a functional food ingredient to control various oxidative processes (Samaranayaka and Li-Chan 2008; Chapter 3 and Chapter 4). FPH was made through autolysis of fish fillet mince using the high activity of endogenous proteases present in post mortem fish muscle due to a parasitic infection by *Kudoa paniformis* (Samaranayaka and others 2006). One-hour autolysis at 52 °C and pH 5.50 was selected to be the appropriate conditions for producing this FPH with good reproducibility in terms of its antioxidative capacity (Samaranayaka and Li-Chan 2008). Fractionation of antioxidative peptides using ultrafiltration (10, 3, and 1 kDa molecular weight cut-offs) further revealed that the size of antioxidative peptides present in FPH were mainly in the range of 1-3 kDa (Chapter 4).

[1]A version of this chapter will be submitted for publication. Samaranayaka AGP, Li-Chan ECY. Identification of antioxidative peptides present in a fish protein hydrolysate produced by an autolysis of Pacific hake (*Merluccius productus*).

However, more detailed characterization of these antioxidant peptides is required in order to conduct additional studies that will confirm *in vivo* antioxidative efficacy, and moreover the antioxidative mechanisms that underly the bioactivity of these constituent peptides. In the present study, various chromatographic techniques and amino acid composition analysis were used to concentrate, isolate and examine the characteristics (i.e. peptide and amino acid profiles) of antioxidative peptides derived from Pacific hake FPH. Selected peptide fractions were subjected to liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) in order to identify antioxidative peptide sequences.

5.2 Materials and Methods

5.2.1 Materials

FPH used in this study was prepared according to the same procedure described in Chapter 3 (Sections 3.2.2 - 3.2.5), using the Inf-30 Pacific hake fish mince batch (see Chapter 3 for details). Freeze-dried hydrolysate was stored at -18 ºC in a sealed container until ready for use. Phenylalanine, lactoferricin, α-cyano-135-4-hydroxycinnamic acid (CHCA), butylated hydroxytoluene (BHT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), linoleic acid, ferrous chloride, ammonium thiocyanate, trifluoroacetic acid (TFA), 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH), and copper sulfate were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Butylated hydroxyanisole (BHA) was purchased from ICN Biomedicals Inc., Aurora, Ohio.

5.2.2 Molecular weight profile of FPH

Gel filtration fast protein liquid chromatography (GF-FPLC) was conducted by loading a 100 μL aliquot of FPH solution (2 mg/mL in distilled deionized water) onto a Superdex Peptide 10/300 GL gel filtration column (10 mm x 300-310 mm, i.d., Amersham Bioscience) that was equilibrated with 30 % acetonitrile containing TFA (0.05%, v/v) and connected to a FPLC system (Amersham Bioscience). The sample was eluted using the same buffer at a

flow rate of 0.2 mL/min, and elution was monitored at 214 nm. Phenylalanine (FW 165) and lactoferricin (FW 3196) were used as molecular weight standards.

The same FPH sample used for GF-FPLC was further analyzed at the Laboratory of Molecular Biophysics Proteomics Core Facility at the University of British Columbia using matrix assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometry in order to obtain information on mass profile of peptides present in the sample. Briefly, a saturated solution of CHCA in 50:50 acetonitrile: 0.1 % TFA in D.D H_2O (v/v) was used as the MALDI matrix. Samples reconstituted in 0.1% TFA were serially diluted (1:1, 1:10 and 1:100) in the CHCA matrix, and 1.0 μL of each dilution was deposited on a stainless steel sample target and allowed to dry at room temperature (dried droplet method). MALDI analysis were performed using Voyager-DE STR delayed-extraction time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA) in the positive ion reflector and/or linear modes. The instrument was equipped with a nitrogen laser (337 nm) to desorb and ionize the samples. Spectra were obtained over the mass acquisition range of 50–500 Da in the reflector mode and 500–10,000 Da in the linear mode, with 50–100 laser shots per spectrum and 20,000 V voltage. A low mass gate was set at 500 Da for analysis of the samples in linear mode to avoid interfering peaks attributed to the CHCA matrix. Mass accuracy is specified at 50 ppm.

5.2.3 Separation and identification of antioxidative peptides from FPH

5.2.3.1 Immobilized metal affinity chromatography (IMAC)

Chelating Sepharose Fast Flow resin (Amersham Bioscience) was packed into a column (1.5 cm x 10 cm, i.d., Bio-Rad Laboratories Inc.) and charged with a 0.05 M $CuSO_4$ solution. The column was equilibrated using 0.02 M phosphate buffer (PB, pH 7.0 with 0.1 M NaCl). FPH sample (0.3 g in 15 mL PB) was loaded onto the column and eluted using the same equilibration PB at pH 7.0 (135 mL), pH 4.0 (75 mL), pH 3.5 (30 mL), and pH 3.0 (75 mL). Elution was monitored spectrophotometrically (Shimadzu UV-1700, Suzhou Instrument Manufacturing Co. Ltd, China) at 280 nm. Peptide fractions were collected, and corresponding fractions were pooled from three replicate chromatography runs. The pooled

fractions were desalted (Micro Acilyzer S1 with Neosepta cartridge AC110-20, ASTOM Corporation, Japan), lyophilized, and stored at -18 ºC until used.

Antioxidative capacity of the IMAC fractions were assayed by measuring the ABTS radical scavenging capacity using the TEAC assay (Chapter 3) at 66.67 μg/mL final concentration. The assay was conducted in both 5 mM PB (pH 7.4 with 0.15 M NaCl) and 75 % ethanol media. Whole FPH and Trolox (15 μM) were used as control samples.

5.2.3.2 Ultrafiltration (UF)

A portion of FPH was fractionated further using ultrafiltration (UF, Amicon® Model 8400, Millipore Corporation, Billerica, MA) with Millipore membranes having molecular weight cut-offs of 10, 3, and 1 kDa as described in Chapter 4 (Section 4.2.4).

FPH and UF fractions were assessed for antioxidative capacities using the linoleic acid peroxidation system (Chen and others 1996). Hydrolysate samples were first dissolved in 1.0 mL of 0.1 M PB (final assay concentrations between 0.001-0.2 mg/mL) in a glass test tube (10 mL volume), followed by the addition of distilled water (0.5 mL) and 50 mM linoleic acid in absolute ethanol (1.0 mL). BHA and BHT control samples were first dissolved in absolute ethanol containing 50 mM linoleic acid (final assay concentrations between 0.001-0.2 mg/mL), followed by the addition of 0.1 M PB (1.0 mL) and distilled water (0.5 mL). The tubes were sealed tightly, vortexed, and kept at 60 °C in the dark in a shaking incubator. Aliquots (50 μL) of the reaction mixtures were withdrawn after 22 h of incubation for the measurement of antioxidative activity using the ferric thiocyanate method (Mitsuda and others 1966). The reaction mixture (50 μL) was mixed with 75 % ethanol (2.35 mL), 30 % ammonium thiocyanate (50 μL), and 20 mM ferrous chloride solution in 3.5 % HCl (50 μL). After 3 min, the absorbance of the colored solution was measured at 500 nm. The percent inhibition of lipid peroxidation was measured using the following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

where control is the reaction mixture without adding any antioxidant sample.

5.2.3.3 Cation-exchange chromatography

A portion of the freeze-dried 1-3 kDa UF fraction was dissolved in sodium acetate buffer (20 mM, pH 4.0) and filtered through a 0.45 μm syringe filter. The filtrate was loaded onto a SP-Sephadex C-25 cation exchange column (2.5 x 40 cm) adequately equilibrated with the same buffer. Fractions were eluted using the equilibration buffer containing NaCl (0.25, 0.5, and 1.0 M) at a flow rate of 2 mL/min, and 4 mL fractions were collected. Absorbance was monitored at 280 nm and the fractions eluted within each peak were pooled, freeze-dried, desalted and tested for ABTS radical scavenging capacity using the TEAC assay (Chapter 3) at 66.67 μg/mL assay concentration.

5.2.3.4 Size exclusion chromatography

Two pooled fractions collected from the ion-exchange column with high antioxidative capacity were purified further using a Sephadex G-25 size exclusion column (1.5 x 73 cm). Each fraction was dissolved in distilled water, loaded onto the column, and eluted with distilled water at a flow rate of 0.5 mL/min. Eluted fractions (2 mL) were pooled into several fractions based on peaks observed by absorbance at 214 nm and freeze-dried. Antioxidative capacity of fractions was measured using the TEAC assay (33.33 and 66.67 μg/mL sample concentrations) and the ORAC assay (four different concentrations between 1 and 250 μg/mL) (Chapter 3).

5.2.3.5 Reversed-phase high performance liquid chromatographic (RP-HPLC) separation and identification of antioxidative peptides

A 5 mg/mL solution of the peptide fraction with highest antioxidative capacity from each of the two gel filtration chromatographic runs, as described above, were prepared in HPLC-grade water and injected onto a Phenomenex Jupiter^{TM} C12 Proteo (4μ, 90 Å) RP-HPLC column (250 x 4.6 mm) at 40 °C. A linear gradient of acetonitrile (0-50 % v/v, in 50 min) and 0.05 % TFA in HPLC-grade water was maintained at a flow rate of 0.5 mL/min and several fractions were collected based on absorbance at 214 nm. The procedure was repeated 20 times to collect sufficient sample for subsequent antioxidative assays and further purification purposes. Pooled fractions from the 20 HPLC runs were freeze-dried after

removing acetonitrile and TFA under a stream of nitrogen, and reconstituted in HPLC-grade water. To facilitate a comparison of relative antioxidative activity for the fractions, appropriate dilutions were made for each fraction depending on the relative amount recovered. Antioxidative capacity was measured using the TEAC assay (Chapter 3).

Peptide fractions with the highest activity were loaded again on to a C-18 RP-HPLC column (75μm x 150mm PepMap, LC Packings, Amsterdam, Netherlands) equilibrated with solvent A containing 0.1 % formic acid and 2 % acetonitrile in water, and eluted with a gradient of 2-40 % of solvent B containing 1 % formic acid and 85 % acetonitrile in water over the course of 60 min at a flow rate of 0.2 mL/min. Mass spectra were recorded using a AB MDS-SCIEX API QSTAR Pulsar *i* mass spectrophotometer (Sciex, Thornhill, ON). The time of flight (ToF) mass analyzer was scanned over a mass-to-charge ratio (*m/z*) range of 300-2000 AU, with a step size of 0.1 AU and the scan time of 1 second. The ion source potential was set at 2.2 KV; the orifice energy was 50 V. To determine the amino acid sequences, the mass spectrometer was operated in an IDA (Information Determined Acquisition) MS/MS mode. Acquisitions were done in Data Directed Acquisition mode (DDA) while a 1 second survey scan was first done from 350 to 1600 *m/z*. The four most intense doubly and triply charged ions were selected to undergo MS/MS fragmentation in 1 second scans from 50 to 2000 *m/z*. The collision energies were determined automatically by the instrument based on the *m/z* values and charged states of the selected peptides.

5.2.3.6 Amino acid analysis

Whole FPH, UF fractions, and semi-purified antioxidative peptide fractions obtained from the chromatographic separations mentioned above were analyzed for total amino acid content by the Advanced Protein Technology Center at The Hospital for Sick Children (Toronto, ON). Amino acid analysis was conducted by first digesting samples with 6 M HCl with 1% phenol @ 110 ^{o}C for 24 h, followed by precolumn derivatization with phenylisothiocarbamate prior to analysis of the phenylthiocarbamyl amino acids on a Waters Pico-Tag HPLC system. The results were expressed as g/100 g of total amino acid content. Performic acid oxidation was performed before determining the Cys content, whereas methanesulfonic acid hydrolysis was used in determining the Trp content.

5.2.4 Statistical analysis

All analyses, except the TEAC assay for HPLC fractions, were performed in triplicate. Statistical analyses were performed using the SPSS statistical program (SPSS 10.0, SPSS Inc., Chicago, IL). Tukey's test was used to evaluate differences among mean values for treatments at the $p< 0.05$ significance level.

5.3 Results and Discussion

5.3.1 Molecular weight distribution profile of FPH

Results from GF-FPLC and MALDI-ToF-MS analysis of FPH are shown in Figures 5.1 and 5.2, respectively. Most of the peptides present in FPH were eluted between the 3200 and 165 Da molecular weight standards (Figure 5.1). Since the unfractionated FPH is a crude mixture of many peptides and free amino acids, the MALDI-ToF-MS spectrum was complex (Figure 5.2). However, this spectrum also confirmed the fact that the majority of the peptides present in FPH are less than about 3000 Da in size.

5.3.2 Concentration of antioxidative peptides from FPH using IMAC

Several studies reported in the literature on antioxidative capacity of protein hydrolysates and peptides indicated that some peptides that have the ability to act as free radical scavengers also have the ability to bind to metal ions such as Cu^{2+}, Zn^{2+}, and Fe^{2+} (Chen and others 1998; Tsuge and others 1991). This is mainly due to the presence of the imidazole group in the His residue found in many of the antioxidative peptide sequences reported in literature, that can act as a hydrogen donor, peroxy radical trapper, or a metal ion chelator (Chan and Decker 1994; Chen and others 1998). It was therefore decided to pass the protein hydrolysates through an IMAC column loaded with Cu^{2+} ion to concentrate antioxidative peptides from FPH made during the present study. It should however be noted that even though the IMAC column was used to concentrate antioxidative peptides due to the possibility of having His residues in antioxidative peptide sequences, the aim was to concentrate peptides that have potential to act as radical scavengers. This is due to the fact

that previous work conducted using this FPH indicated that the antioxidative mechanism is mainly due to the ability of constituent peptides to act as hydrogen or electron donors rather than as ion chelators (Chapter 3).

Peptides were eluted from the IMAC column as four fractions recovered at pH 7, 4, 3.5, and 3, and labeled accordingly as I-1, I-2, I-3, and I-4, respectively (Figure 5.3). The percentage yield of each fraction after desalting and freeze-drying was 62.6 %, 16.1 %, 10.4 %, and 10.9 % for I-1, I-2, I-3, and I-4, respectively. The majority of components present in whole FPH did not bind to the IMAC column and were eluted in I-1. However, there were some peptides, and/or free His, in the FPH that could bind to Cu^{2+} and only eluted at a lower pH. The I-1 peptide fraction, which was eluted at pH 7 without binding to the IMAC column, possessed the highest ABTS radical scavenging capacity (Figure 5.4). ABTS radical scavenging capacity of whole FPH was higher than that of I-1 fraction (Figure 5.4) indicating possible synergistic effects of constituent peptides when present in the whole FPH.

The IMAC fractions I-2, I-3, and I-4 were not completely soluble in PB used during the previous TEAC assay and therefore the assay was repeated by dissolving IMAC fractions and ABTS radical cations in 75 % ethanol. All fractions were completely dissolved in 75 % ethanol. The ABTS radical scavenging capacity of I-1 and I-2 fractions, however, decreased in 75 % ethanol media whereas the radical scavenging capacity of I-3 and I-4 were not different compared to the results obtained by conducting the assay in PB (Figure 5.4). It was therefore clear that the poor solubility of the peptide fractions was not the reason for having lower ABTS radical scavenging ability for I-2, I-3, and I-4 IMAC fractions. Considering these findings, it was decided to apply other methods than immobilized metal ion-chelating columns in order to concentrate antioxidative peptides from Pacific hake FPH.

5.3.3 Concentration of antioxidative peptides from FPH using UF

Pacific hake FPH made through autolysis was subjected to UF during previous work (Chapter 4) and the results indicated that antioxidative peptides present are mainly in the mass range of 1-3 kDa (Table 4.1, Chapter 4). The TEAC assay and the ORAC assay were used in analyzing UF fractions for antioxidative capacity during this previous study. It was therefore decided to use UF to first concentrate antioxidative peptides from FPH during the present study. Further, linoleic acid peroxidation system was used to assess antioxidative

capacity of UF fractions made during current study aiming to compare the results obtained from previous work by performing the TEAC and ORAC assays for UF fractions (Chapter 4).

Yields of the UF fractions were 7.19 %, 26.68 %, 17.63 %, and 44.03 % (as percentage of the weight of freeze dried FPH used for UF separation) for >10 kDa, 3-10 kDa, 1-3 kDa, and <1 kDa, respectively. In the linoleic acid peroxidation system, sample concentrations below 0.05 mg/mL were not effective at controlling lipid oxidation reactions, except for the whole FPH which showed low levels (~12 %) of inhibition at 0.025 mg/mL (Table 5.1). The amounts of antioxidative peptides present in the system at these lower sample concentrations might have been insufficient to scavenge free radicals and inhibit lipid autoxidation processes. It is hard to explain why some of these samples acted as prooxidants at lower assay concentration. Even BHT resulted in negative % inhibition value at 0.001 mg/mL assay concentration. However, both BHA and BHT possessed some lipid autoxidation inhibitory power even at 0.01 mg/mL and showed a dose-dependent increase up to 0.05 mg/mL. The reason for not showing dose-dependency above 0.05 mg/mL for BHT was that it could scavenge almost all peroxide radicals available in the system at this concentration (Table 5.1). The 1-3 kDa UF fraction also did not show a significant ($p > 0.05$) increase in its activity when the concentration was increased from 0.1 to 0.2 mg/mL. However, a dose dependent inhibition of lipid peroxidation by whole FPH was observed with the increase of concentration from 0.1 to 0.2 mg/mL, and the inhibition by FPH at 0.2 mg/mL was not significantly different from 1-3 kDa UF fraction at 0.2 mg/mL. Previous research indicated that the lipid peroxidation inhibitory-capacity of this FPH in a linoleic acid peroxidation system (at 0.2 mg/mL assay concentration) was higher ($p < 0.05$) than that of BHA and α-tocopherol over prolonged storage (~162 hrs) (Samaranayaka and Li-Chan 2008 and Chapter 3). Therefore FPH and/or 1-3 kDa UF fraction could be useful in controlling lipid oxidation in emulsion-type food and possibly in biological systems where lipid/water bilayers are exposed to or in contact with oxidative agents.

As previously mentioned, results from TEAC assay and ORAC assay (Chapter 4) also indicated that the ultrafiltration concentrated antioxidative peptides recovered from Pacific hake FPH were present mainly in the 1-3 kDa UF fraction. Antioxidative peptides identified from other fish sources in the literature were also reported to have molecular weights

between 500 and 1800 Da (Wu and others 2003; Jun andothers 2004; Je and others 2005a,b; Ranathunga and others 2006). For example, a 1-3 kDa peptide fraction from a hoki frame protein hydrolysate prepared by pepsin hydrolysis had comparable antioxidative activity to that of α-tocopherol in a linoleic acid emulsion system (Je and others 2005a). A peptide with molecular weight of approximately 1400 Da from a mackerel protein hydrolysate possessed a strong *in vitro* antioxidative activity (Wu and others 2003).

Based on these findings, the 1-3 kDa UF fraction was selected for further purification of antioxidative peptides using various chromatographic techniques as discussed below.

5.3.4 Cation-exchange chromatography

As shown in Figure 5.5, thirteen fractions were collected from cation-exchange chromatographic separation of the 1-3 kDa UF fraction. When these fractions were tested for ABTS radical scavenging capacity, fraction F-12 followed by F-10 possessed the strongest radical scavenging capacity compared to the other fractions (Figure 5.6). The fraction F-10 was eluted with sodium acetate buffer (pH 4.0) with 0.25 M NaCl, whereas the fraction F-12 was eluted with the buffer with 0.5 M NaCl. Therefore, peptides present in F-12 should be more cationic at pH 4.0 compared to the peptides eluted in the F-10 fraction. Approximate yields of desalted and freeze dried F-10 and F-12 fractions based on the freeze dried weight of 1-3 kDa UF fraction were 9 % and 14 %, respectively.

5.3.5 Size exclusion chromatography

Both F-10 and F-12 fractions from cation-exchange column were further separated by size-exclusion chromatography (Figures 5.7a and 5.8a). For both samples, the smaller molecular weight fraction (F-10.3 and F-12.3) contained most of the antioxidative peptides (Figures 5.7b and 5.8b), although antioxidative activity was widely observed for all fractions. ORAC values for F-10.3 and F-12.3 were also determined to be 871.2 ± 7.5 μmol Trolox/g and 703.3 ± 36.9 μmol Trolox/g, respectively. Compared to the antioxidative capacity of 1-3 kDa UF fraction (ORAC value of 330 ± 18 μmol Trolox/g, Chapter 4 Table 4.1), these two semi-purified peptide fractions possessed significantly ($p<0.05$) higher antioxidative potential. Since the amounts of peptides collected after size-exclusion chromatographic

separation were very small, quantification of the peptides present in F-10.3 and F-12.3 fractions was not possible.

5.3.6 RP-HPLC Separations

F-10.3 and F-12.3 fractions were further subjected to RP-HPLC on a Jupiter^{TM} Proteo 90 Å peptide column. The resultant chromatograms and ABTS radical scavenging assay results are shown in Figures 5.9 (a,b) and 5.10 (a,b) for F-10.3 and F-12.3 fractions, respectively. The area under each HPLC peak was used to estimate the relative amounts of peptides present in each fraction since it was very difficult to collect enough peptides to get actual sample weights. Fractions F-10.3.5 and F-12.3.2 had high antioxidative activities (Figures 5.9 (b) and 5.10 (b)) compared to other pooled fractions and were therefore selected for further purification using RP-HPLC on a C-18 column. It should however be noted that for both samples, antioxidative activity of selected fractions was not significantly different from several other HPLC fractions that also had fairly high ABTS radical scavenging potential (Figures 5.9 (b) and 5.10 (b)). This selection process may miss some important antioxidative peptides present in F-10.3 and F-12.3 fractions during the peptide purification and identification process, especially because the assay concentrations of peaks were an "estimation" based on the HPLC peak area.

Chromatograms obtained by C-18 RP-HPLC separation and ToF-MS detection of peptide ions from F-10.3.5 and F-12.3.2 fractions over time are shown in Figures 5.11 and 5.12, respectively. Peaks selected for identifying peptides by MS/MS analysis, and MS/MS analysis results are also shown in those Figures. Two peptide sequences could be identified from F-12.3.2 fraction. They were, Pro-Leu-Phe-Gln-Asp-Lys-Leu-Ala-His-Ala-Lys (1266 Da) (A) and Ala-Glu-Ala-Gln-Lys-Gln-Leu-Arg (1125 Da) (B). **Appendix III** shows matching of predicted and actual ion peaks obtained from MS/MS analysis in identifying peptides A and B. The highlighted ones are the actual ion peaks obtained. Fifty one percent of the ions were matched for peptide A, whereas 82 % matching ions were found for peptide B (**Appendix III**). Identifying peptide sequences from F-10.3.5 fraction was however not successful. One reason for the difficulty in identifying peptide sequences from the autolytic digest of Pacific hake fish mince could be the difficulty in matching those sequences with the

available peptide sequences in databases used. To date, protein sequences of Pacific hake are not available in protein databases such as Swissprot and Genbank. Furthermore, the identification of sequences is based on matching the unknown sequences of hake peptides arising mainly from autolytic digestion by cathepsin L-like proteases, with those in databases which are predominantly peptides released by action of proteases like trypsin.

5.3.7 Amino acid composition

Even though the antioxidative activity was different for FPH and different UF fractions (Table 5.1 and Table 4.1 in Chapter 4), the total amino acid compositions were relatively similar (Table 5.2). Only <1 kDa UF fraction contained relatively higher amount of Leu and Cys and lower amount of Glx. Thus, rather than the total amino acid composition of the whole fraction, the variation of the peptide sizes, sequences, and free amino acid contents in each sample are likely responsible for these differences in antioxidative potential of UF fractions. The Asx and Glx contents in F-10.3 and F-12.3 fractions were higher than the amounts present in 1-3 kDa UF fraction. Further, different amino acid profiles were obtained for F-10.3 and F-12.3 fractions: amounts of Asx, Pro, Ala, Val, Ile, and Leu were higher in F-10.3 whereas amounts of His, Arg, Tyr, Lys, and Cys were higher in F-12.3.

5.4 Conclusions

Identification of antioxidative peptides from Pacific hake FPH could not be completed during the present study due to the fact that it was difficult to match sequences in the available protein databases to the peptides present in relatively pure peptide fractions such as F-10.3.5. As mentioned earlier in Section 5.3.6, there were also several other RP-HPLC fractions that contained high amount of antioxidative peptides after fractionation on the Jupiter Proteo peptide column. Consequently, no further LC-MS/MS analysis was performed on those fractions since it was already difficult to identify peptide sequences from F-12.3.2 and F-10.3.5 fractions.

However, it was possible to concentrate antioxidative peptides into several semi-pure fractions using UF, cation-exchange chromatography, followed by size-exclusion

chromatography during the present work, and also to identify several important characteristics of antioxidative peptides present in FPH. These peptides were mainly within the range of 1-3 kDa molecular mass range and could effectively inhibit the lipid peroxidation in a linoleic acid model system. Yields of the F-10 and F-12 semi-purified fractions based on the original weight of whole FPH were 1.6 % and 2.5 %, respectively. It was however not possible to quantify the yields of more pure antioxidative peptide fractions such as F-10.3, F-12.3, as well as F-10.3.5 and F-12.3.2 due to the small sample sizes. Nevertheless, it was evident from TEAC and ORAC assay results that these fractions had much higher antioxidative potential compared to the whole FPH and 1-3 kDa UF fraction.

Most of the radical scavenging peptides from FPH did not have accessible His-residues in amino acid sequences to facilitate binding to the immobilized Cu^{2+} ion during IMAC column separation, but instead had enough positively charged groups at pH 4.0 to bind to the cation-exchange resin. Amino acid composition analysis of FPH and the 1-3 kDa UF fraction also indicated that these samples contained high amounts of basic amino acid residues such as Lys and Arg. Contents of Asx (Asp+Asn) and Glx (Glu+Gln) were also high in FPH and other UF fractions. Further, peptide A (Pro-Leu-Phe-Gln-Asp-Lys-Leu-Ala-His-Ala-Lys) and peptide B (Ala-Glu-Ala-Gln-Lys-Gln-Leu-Arg) identified from FPH also possess 27 % and 25 % basic residues, respectively, in their sequences. Peptide A contains 27 % hydrophobic residues whereas the hydrophobic residues present in peptide B was only 12 %. Many antioxidative peptides identified in the literature include the hydrophobic amino acid residues Val or Leu at the N-terminus of the peptides and Pro, His, or Tyr in the sequences (Uchida and Kawakishi 1992; Chen and others 1995). Peptide A identified from Pacific hake FPH also contains Leu close to the N-terminus, and Pro and His in its sequence. It would be interesting to know how these molecular characteristics would contribute to the antioxidative action of these two peptides. Further studies are therefore necessary to confirm antioxidative potential of peptides A and B as well as to study the mechanisms of antioxidative action with respective to their size, sequence, and other molecular characteristics mentioned above.

Table 5.1 Inhibition of lipid peroxidation by fish protein hydrolysate (FPH) and ultrafiltration fractions at different sample concentrations in a linoleic acid peroxidation system incubated at 60 °C for 42 h[1]

Sample concentration (mg/mL)	% Inhibition of lipid peroxidation						
	FPH	**>10 kDa**	**(3-10) kDa**	**(1-3) kDa**	**<1 kDa**	**BHA**	**BHT**
0.001	-11.41 ±9.99 *	0.25 ±5.91	-30.51 ±9.07	-4.62 ±19.06 *	-9.55 ±11.89	-5.45 ±13.39 *	-29.29±15.15 *
0.010	-13.33 ±3.82 *	-10.00 ± 4.78	-13.97 ±9.31	-17.50 ±3.35 *	-10.19 ±7.21	10.19 ±4.19 *	13.59 ±6.25 **
0.025	11.86 ±9.64 **	-8.65 ±3.02	-10.38 ±7.34	-6.79 ±8.72 *	-9.81 ±6.19	50.45 ±7.90 **	56.54 ±2.41 ***
0.050	19.42 ±10.78 a,b**	35.22 ±12.64 b	23.55 ±13.17 b	25.51 ±6.20 b**	-4.56 ±3.85 a	79.28 ±5.24 c***	96.38 ±0.63 c****
0.10	45.36 ±3.92 b***	27.54 ±8.04 a	41.38 ±2.01 a,b	70.51 ±7.60 c***	33.40 ±6.67 a,b	87.75 ±2.18 d***	97.97 ±1.27 d****
0.20	75.58 ±2.13 c****	54.28 ±5.77 a	76.16 ±1.76 c	79.86 ±0.55 c,d***	66.95 ±3.62 b	86.23 ±2.38 d***	99.86 ±0.66 e****

[1]Mean ± standard deviation from triplicate analyses. Values within each sample concentration of 0.05, 0.10, and 0.20 mg/mL in a row bearing different letters are significantly different at $p < 0.05$. Differences ($p < 0.05$) in % inhibition of lipid peroxidation by the same sample at different concentrations are also indicated for FPH, 1-3 kDa, BHA, and BHT using different number of "*" symbols. Values with negative sign indicate prooxidant activity.

Table 5.2 Amino acid composition of fish protein hydrolysate (FPH), ultrafiltration fractions, and semi-purified antioxidative peptide fractions

Amino acid	Composition (g/100 g)[a]							
	FPH	**>10 kDa**	**(3-10) kDa**	**(1-3) kDa**	**<1 kDa**	**F-10.3**	**F-12.3**	**F-12.3.2**
Aspartic acid +Asparagine	10.3	11.8	11.5	10.9	9.0	15.2	12.3	13.0
Glutamic acid +Glutamine	19.0	19.7	20.1	19.8	17.0	25.8	27.0	25.2
Serine	4.0	4.0	4.2	4.2	3.9	4.3	3.6	4.1
Glycine	4.5	5.6	4.7	3.6	4.1	4.3	2.6	3.2
Histidine	1.9	1.8	2.1	2.0	1.9	1.3	3.0	2.2
Arginine	8.0	8.0	7.8	7.2	8.4	4.0	9.0	8.8
Threonine	4.0	3.8	3.9	4.2	4.4	4.0	2.9	3.3
Alanine	6.3	6.3	6.4	6.0	6.2	6.1	5.1	5.4
Proline	3.4	4.2	3.8	3.1	2.7	3.6	1.9	1.5
Tyrosine	2.8	2.5	2.7	3.0	3.4	1.9	2.8	3.0
Valine	4.8	4.9	4.8	5.1	4.9	4.7	3.6	2.9
Methionine	3.1	2.1	2.6	3.2	4.0	2.2	1.9	3.6
Isoleucine	3.7	4.0	4.0	3.9	3.4	4.4	3.7	1.7
Leucine	9.8	6.2	7.4	9.8	12.5	8.2	7.4	7.4
Phenylalanine	3.0	2.6	2.5	3.1	3.7	2.1	1.9	0.4
Lysine	10.4	12.3	11.3	9.9	9.1	6.7	10.0	13.3
Cysteine	0.9	ND[c]	ND	0.7	1.2	0.6	1.2	0.8
Tryptophan	0.2	ND	ND	0.2	0.2	0.5	0.2	0.2
Total amino acid content[b]	69.2	97.5	96.8	88.0	53.7	90.9	82.8	

[a]Presented as g/100g total amino acid content of sample

[b]Calculated based on dry matter weight of sample, [c]Not determined

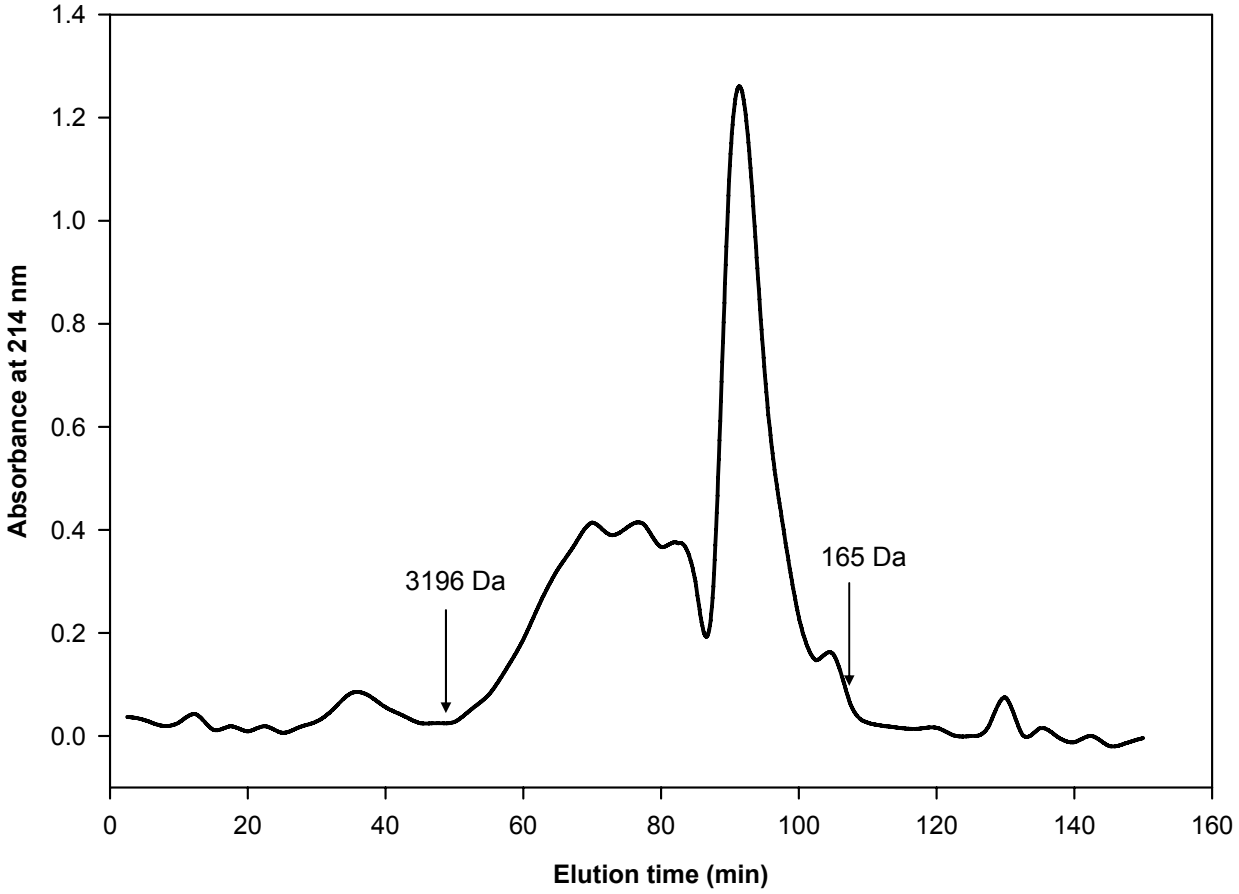

Figure 5.1 Molecular weight distribution profile of fish protein hydrolysate (FPH) obtained using GF-FPLC analysis

Arrows indicate the elution of the molecular weight standards (lactoferricin (3196 Da) and phenylalanine (165 Da)).

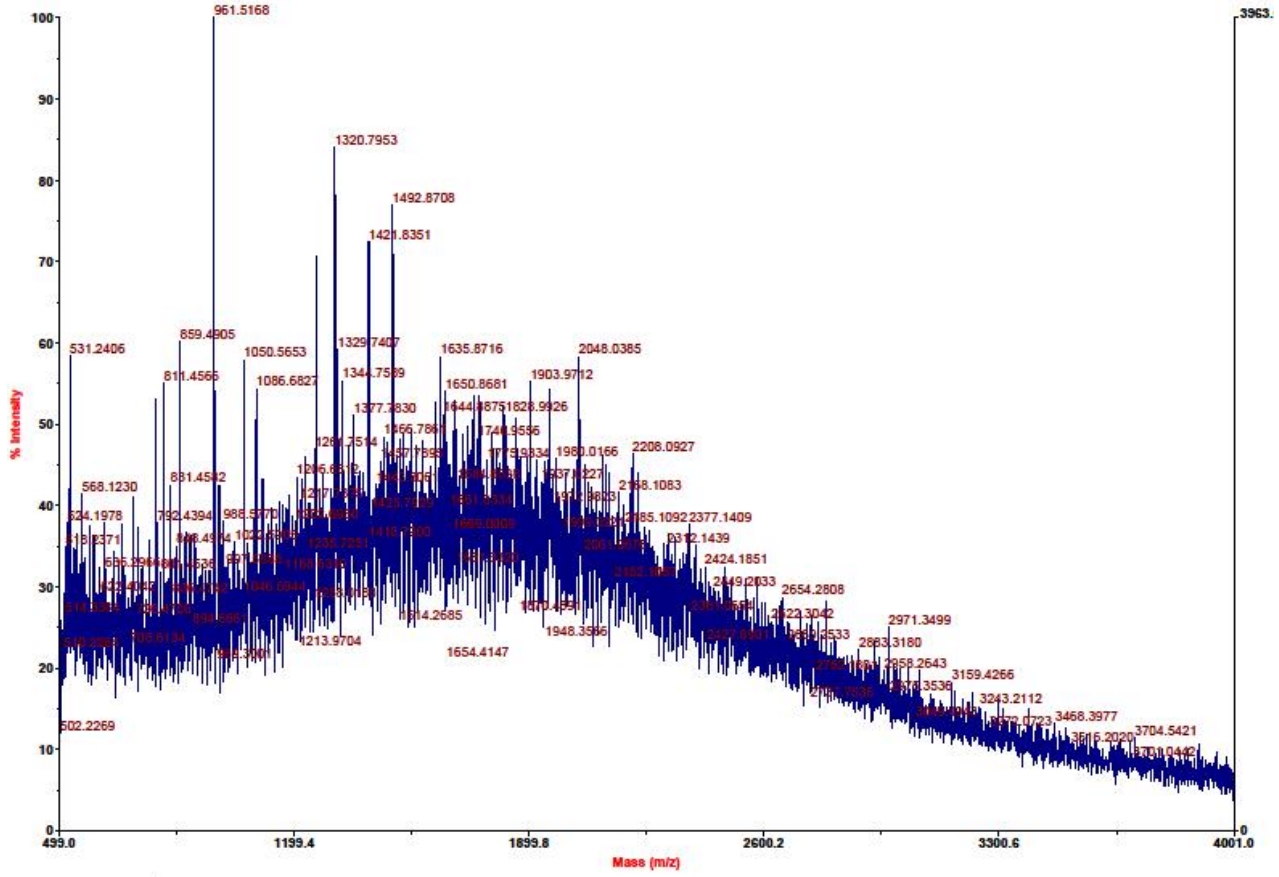

Figure 5.2 Molecular weight distribution profile of fish protein hydrolysate (FPH) obtained using MALDI-ToF-MS analysis

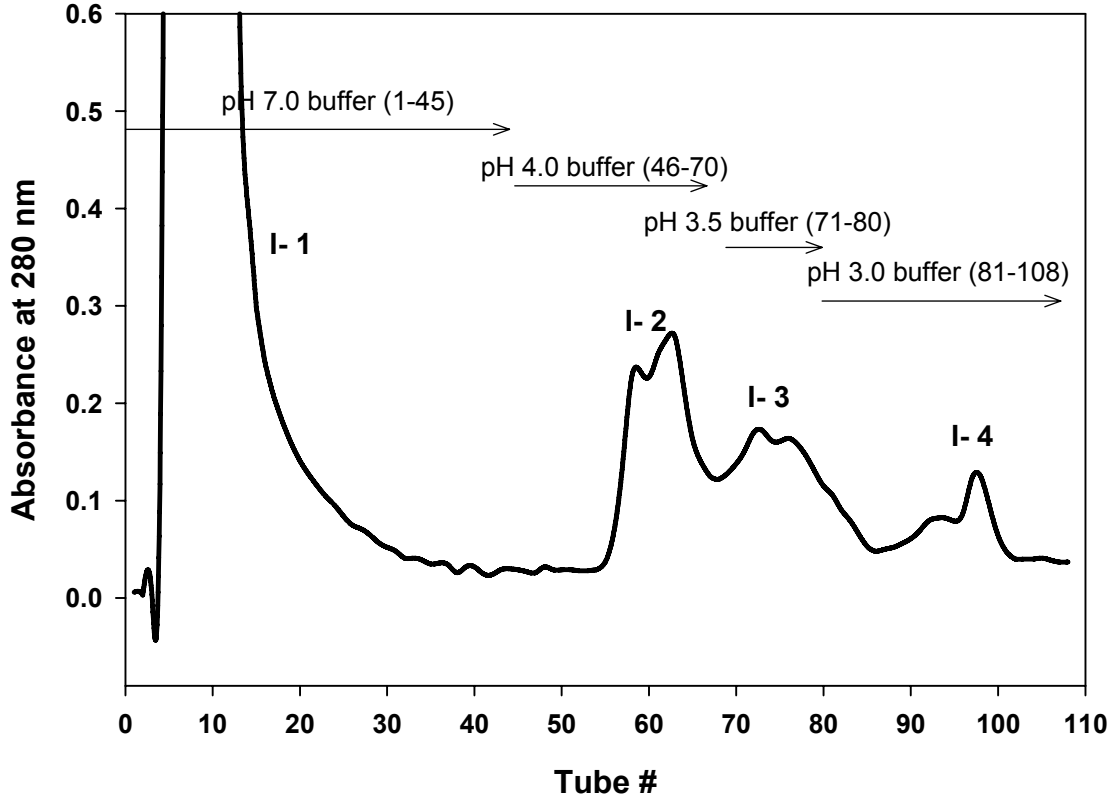

Figure 5.3 IMAC separation of Pacific hake fish protein hydrolysate (FPH)
Tube numbers (4-30), (56-68), (69-85) and (87-100) were pooled into four fractions referred to as I-1, I-2, I-3, and I-4, respectively.

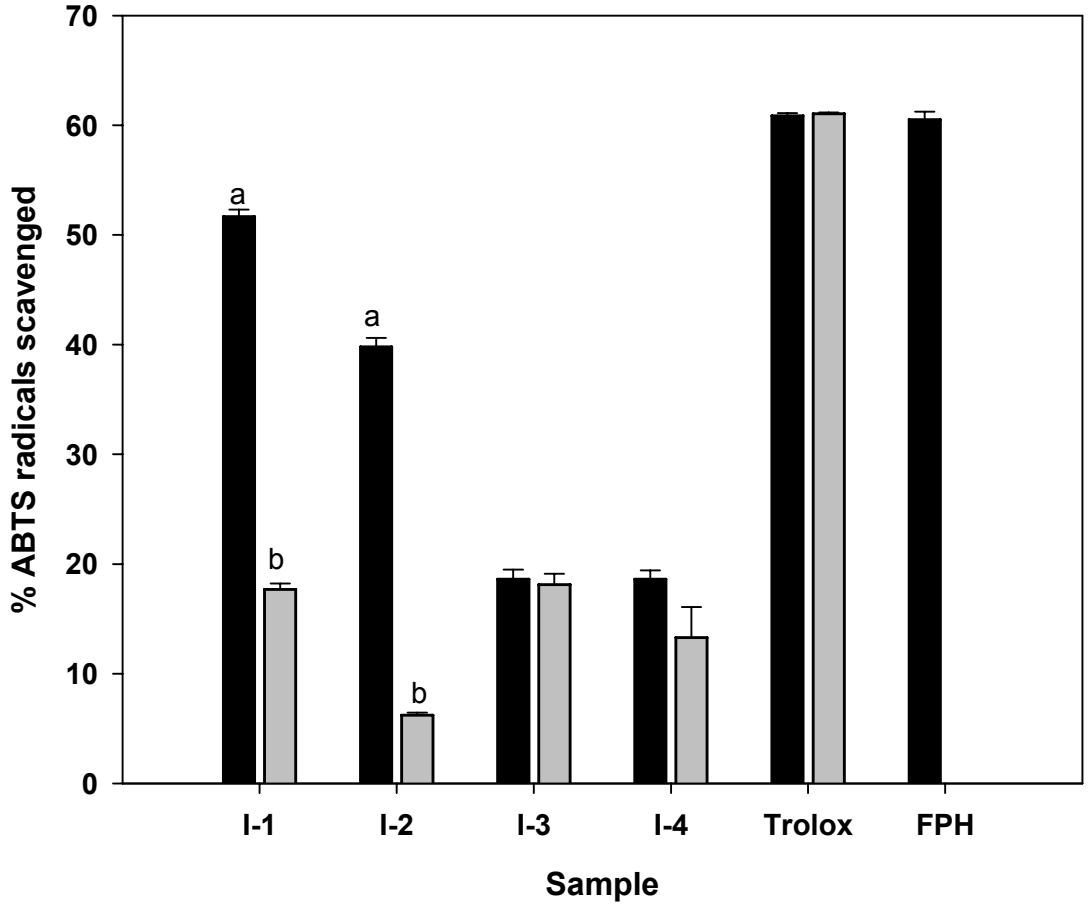

Figure 5.4 ABTS radical scavenging capacity of IMAC fractions (I-1–I-4), Trolox, and whole fish protein hydrolysate (FPH)

Black bars shows results obtained by conducting the trolox equivalent antioxidative capacity (TEAC) assay in phosphate buffer (pH 7.4 with 0.1 M NaCl) whereas gray bars shows results obtained by conducting the assay in 75 % ethanol. Final assay concentrations of samples were 66.67 µg/mL for IMAC samples and FPH, and 15 µM for Trolox.

Mean values from triplicate measurements. Bars with different letters (a,b) within the same sample are significantly different at $p < 0.05$.

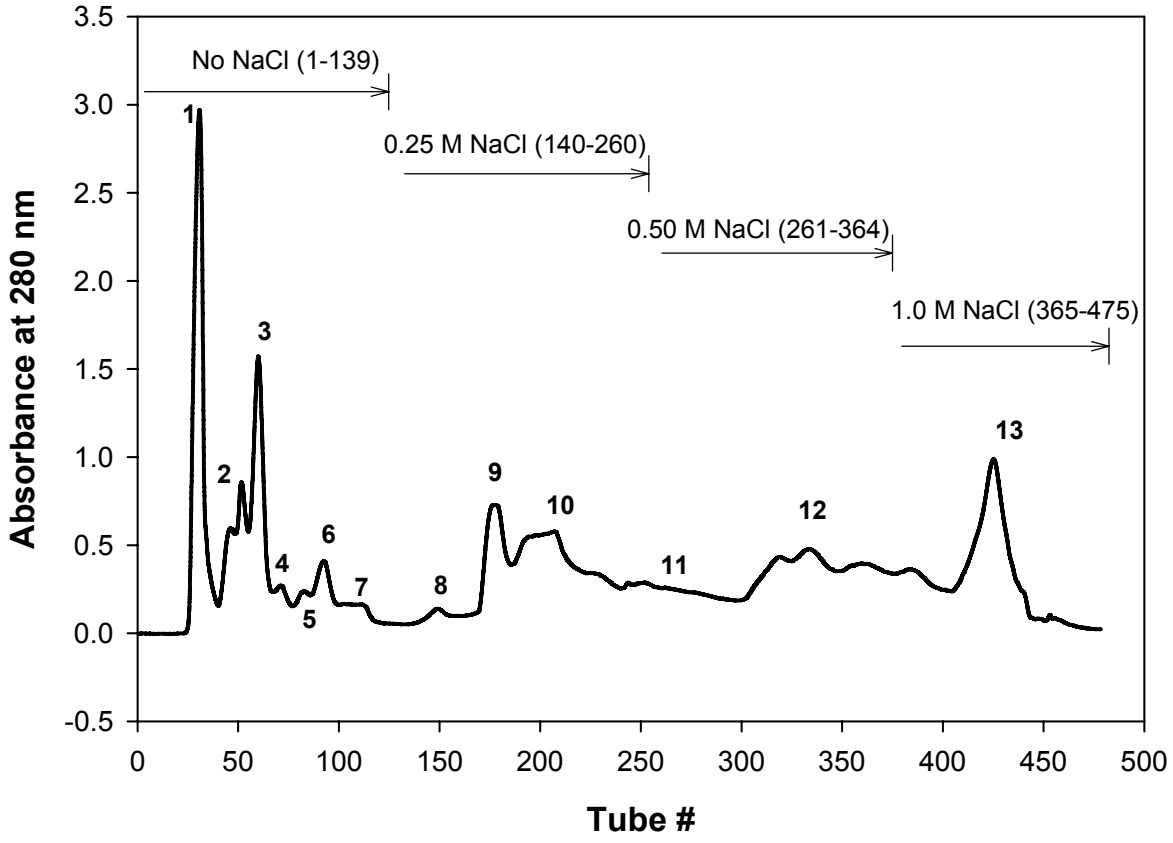

Figure 5.5 SP-Sephadex C-25 cation-exchange column separation of 1-3 kDa ultrafiltration fraction

Fractions were eluted with sodium acetate buffer (20 mM, pH 4.0) with 0, 0.25, 0.50 and 1.0 M NaCl. Tube numbers for the fractions collected to make 1-13 pooled fractions were (25-41), (42-51), (52-67), (68-74), (76-94), (97-116), (119-141), (143-161), (162-177), (178-267), (270-308), (310-368), and (369-427).

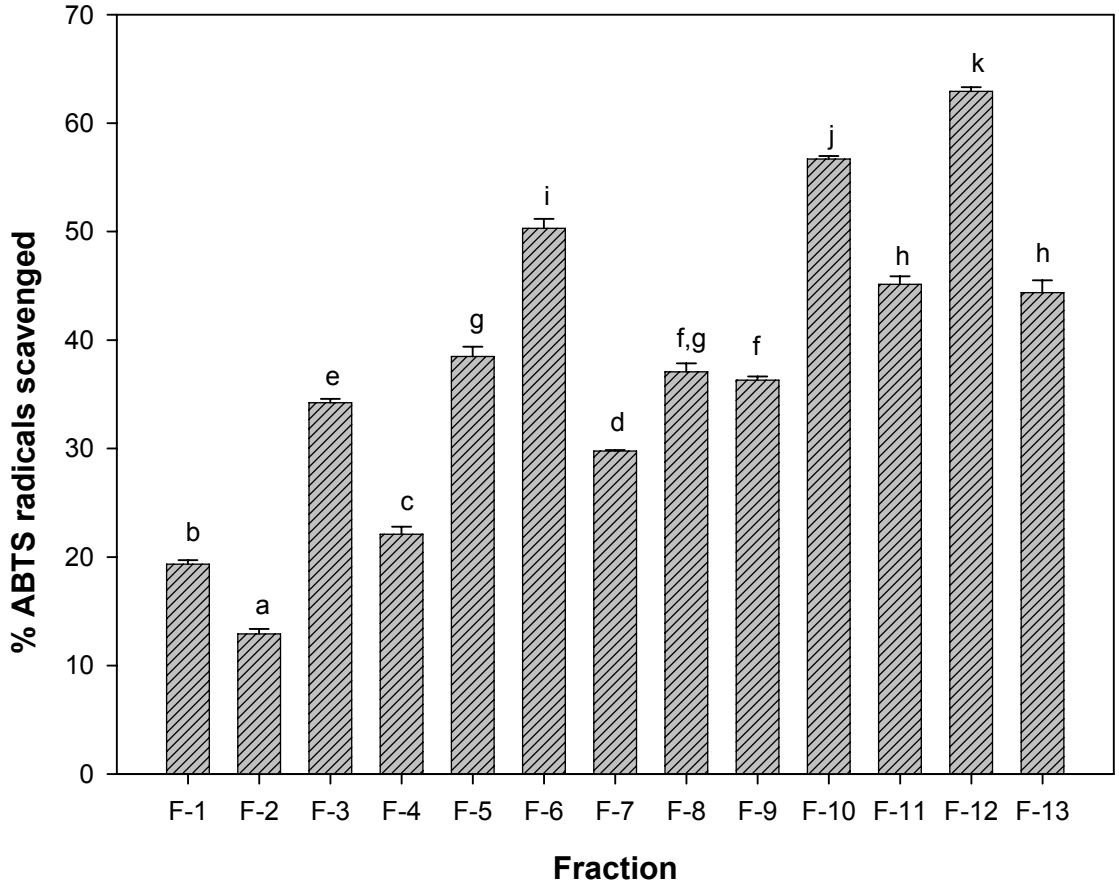

Figure 5.6 ABTS radical scavenging capacity of cation exchange column fractions

Final assay concentrations of samples were 66.67 µg/mL. Mean values from triplicate measurements. Fractions having different letters (a-k) are significantly different at $p < 0.05$.

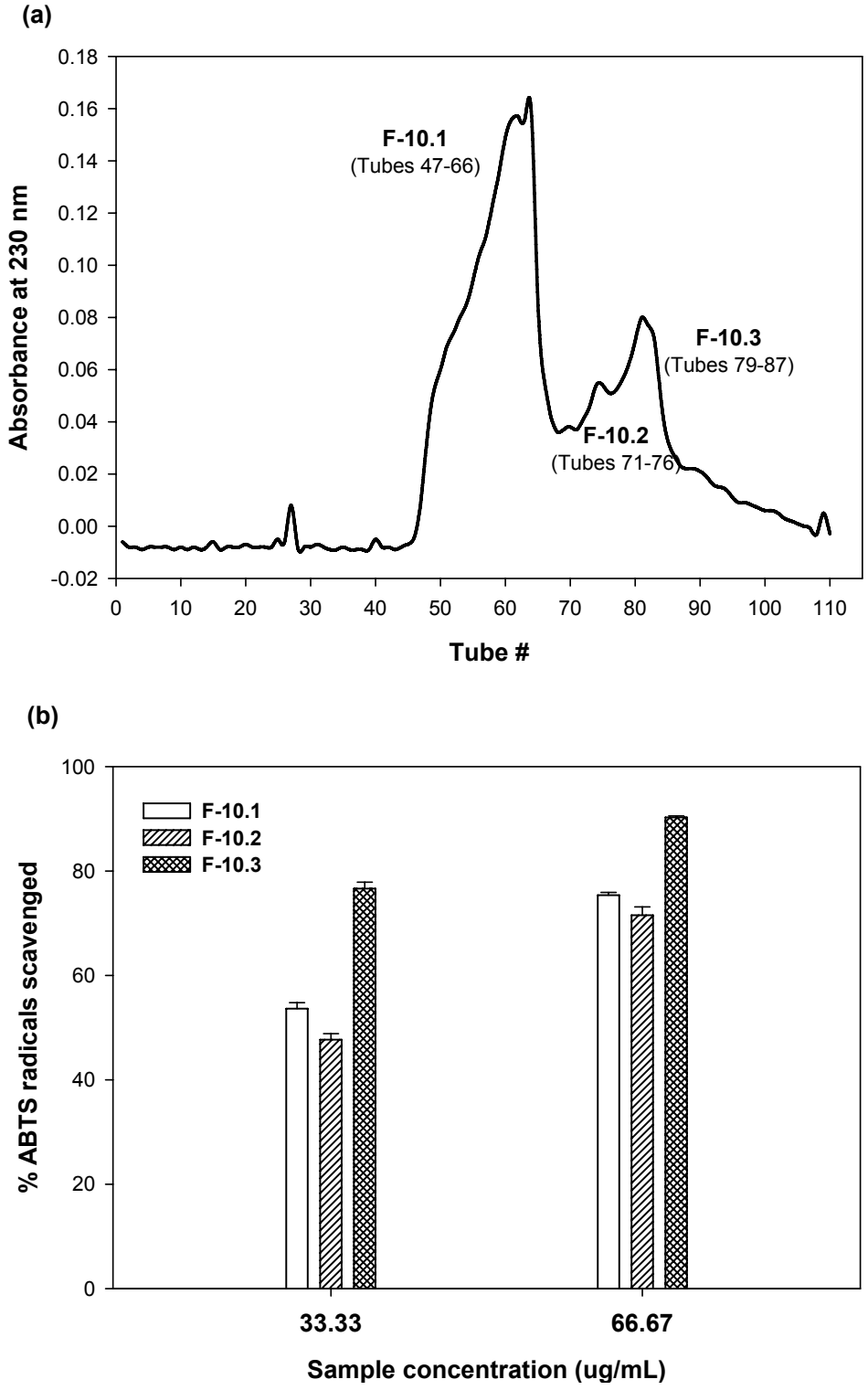

Figure 5.7 Separation of F-10 cation-exchange column fraction using Sephadex G-25 size exclusion column (a) and ABTS radical scavenging capacities (b) of pooled fractions

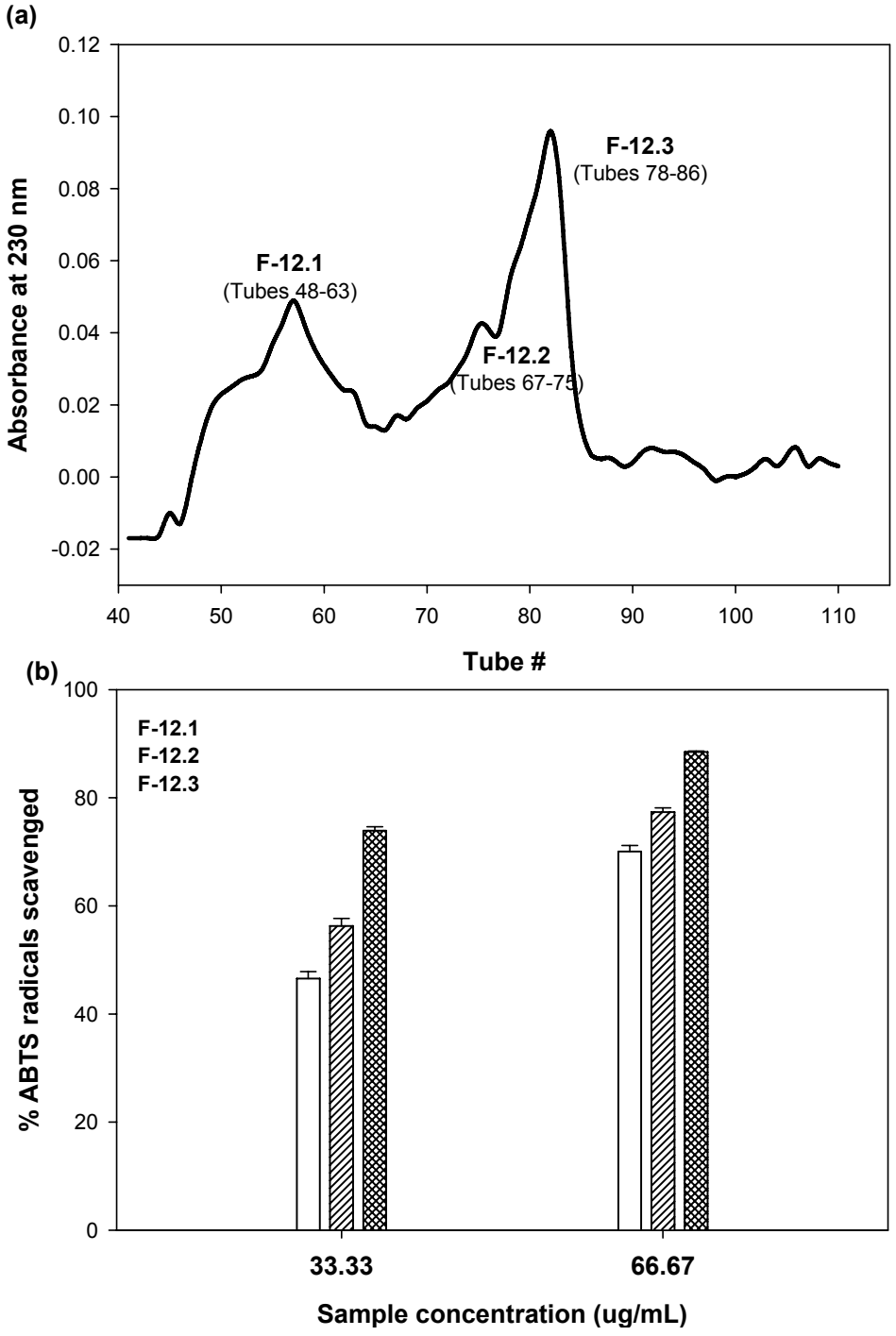

Figure 5.8 Separation of F-12 cation-exchange column fraction using Sephadex G-25 size exclusion column (a) and ABTS radical scavenging capacities (b) of pooled fractions

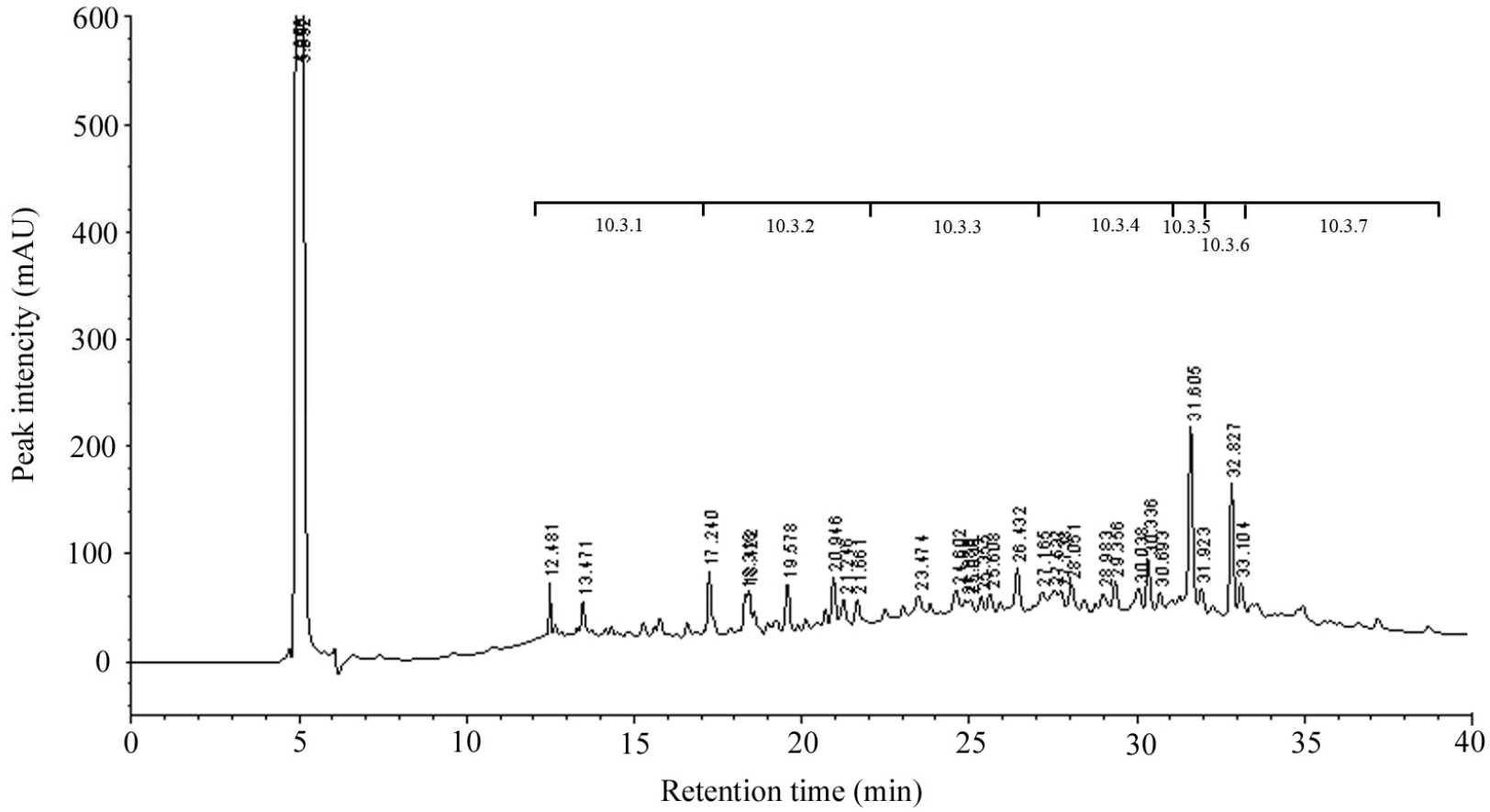

Figure 5.9a RP-HPLC separation of F-10.3 fraction using Jupiter^{TM} Proteo 90 Å peptide column

Retention time ranges for the fractions (F-10.3.1 to F-10.3.7) collected were 12-17, 17-22, 22-27, 27-31, 31-32, 32-33.2, and 33.2-39 min.

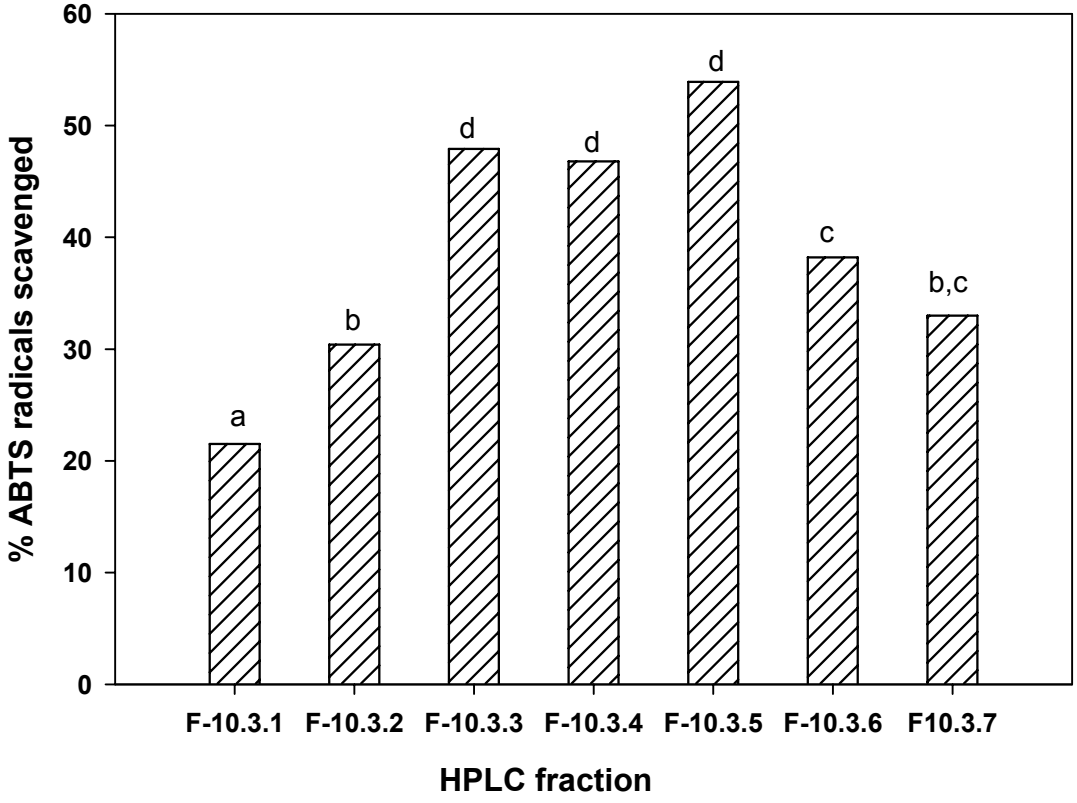

Figure 5.9b ABTS radical scavenging capacity of pooled fractions from RP-HPLC separation of F-10.3 fraction using Jupiter^{TM} Proteo 90 Å peptide column

Mean values from duplicate measurements. Appropriate dilutions were made for each fraction depending on the HPLC peak area, in order to obtain similar peptide concentrations for the assay. Fractions with different letters are significantly different at $p < 0.05$.

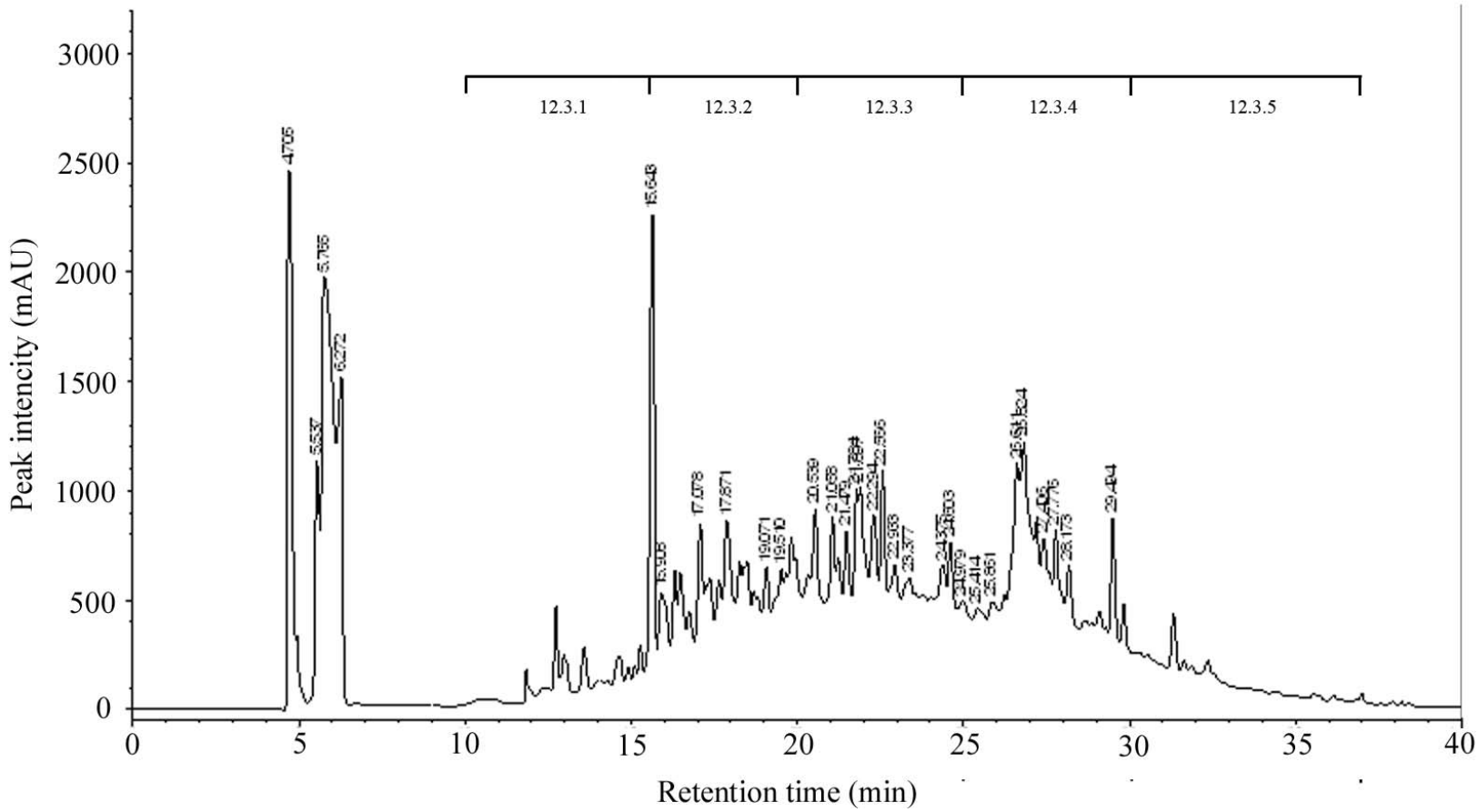

Figure 5.10a RP-HPLC separation of F-12.3 fraction using Jupiter^{TM} Proteo 90 Å peptide column

Retention time ranges for the fractions (F-12.3.1 to F-12.3.5) collected were 10-15.5, 15.5-20, 20-25, 25-30, and 30-37 min.

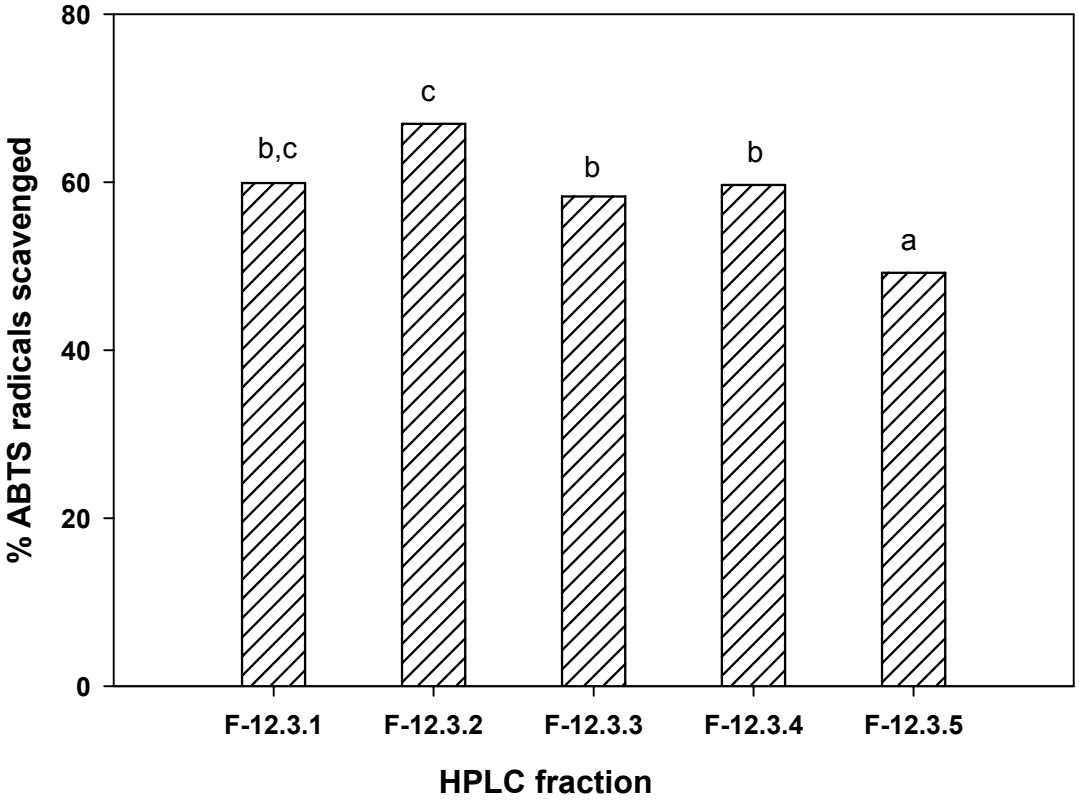

Figure 5.10b ABTS radical scavenging capacity of pooled fractions from RP-HPLC separation of F-12.3 fraction using Jupiter^{TM} Proteo 90 Å peptide column
Mean values from duplicate measurements. Appropriate dilutions were made for each fraction depending on the HPLC peak area, in order to obtain similar peptide concentrations for the assay. Fractions with different letters are significantly different at $p < 0.05$.

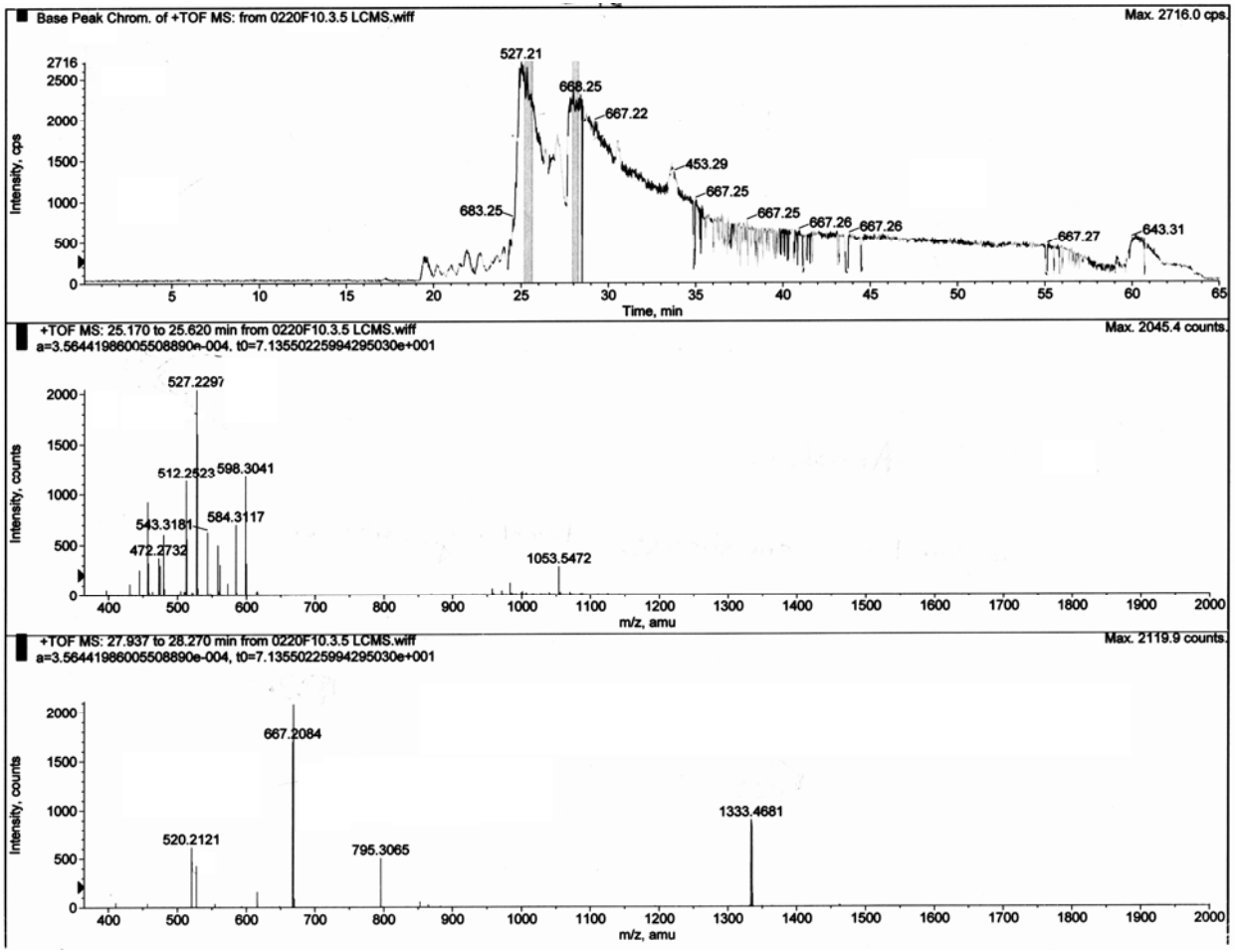

Figure 5.11 Base peak chromatogram from HPLC separation of F-10.3.5 fraction on a C-18 column and detection by ToF-MS

Peaks selected for further MS/MS analysis in order to identify peptide sequences, and subsequent MS/MS analysis are also shown.

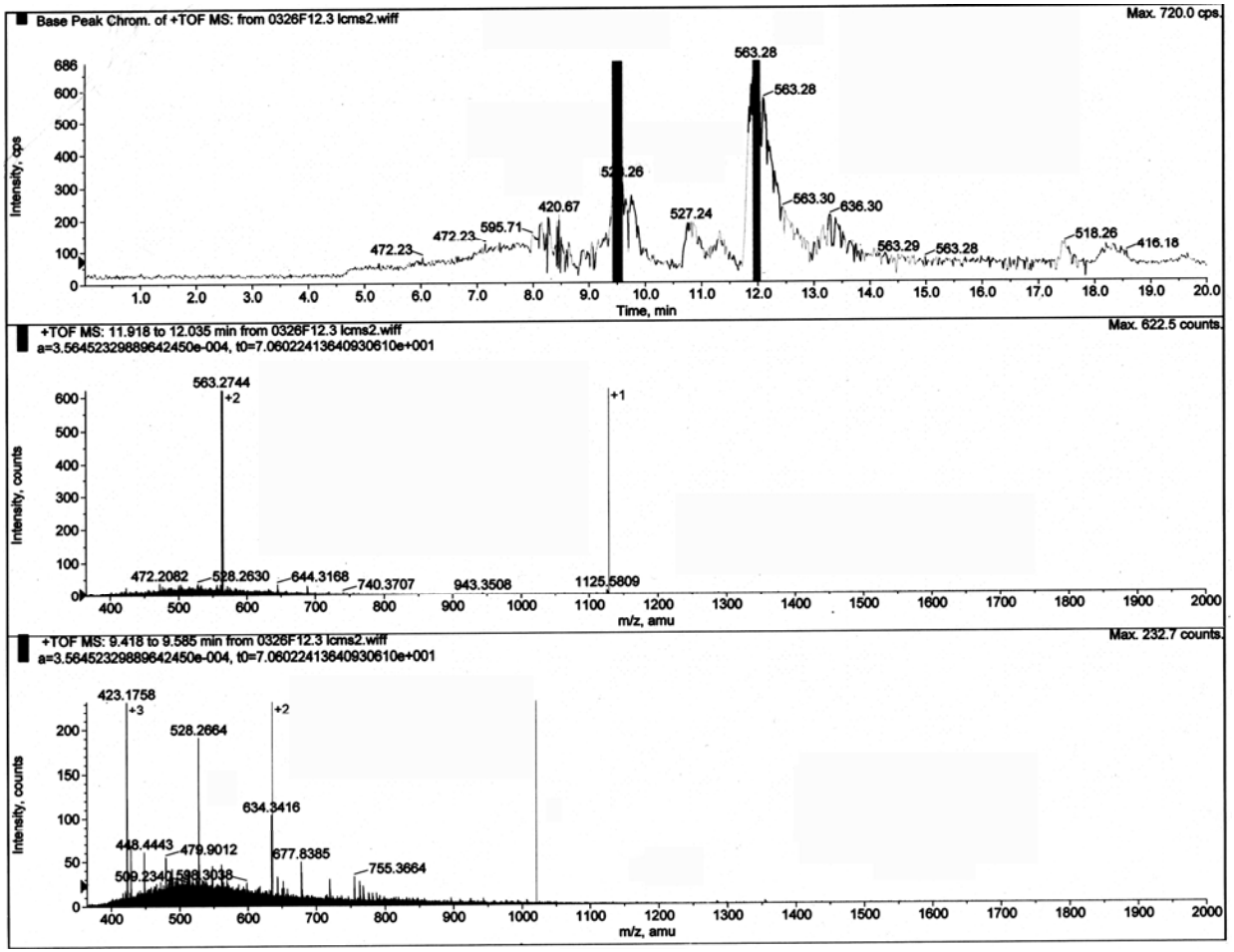

Figure 5.12 Base peak chromatogram from HPLC separation of F-12.3.2 fraction on a C-18 column and detection by ToF-MS

Peaks selected for further MS/MS analysis in order to identify peptide sequences, and subsequent MS/MS analysis are also shown.

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CHAPTER 6 Investigation into Antioxidative Capacity of Peptides Derived From Several Food Sources, Including Pacific Hake, Using Different *In Vitro* Assay Systems[1]

6.1 Introduction

In vitro antioxidant assays based on chemical reactions are widely used in preliminary screening of bioactive compounds that can potentially be used as food or pharmaceutical ingredients. A great deal of attention has appeared in recent literature to identify and assess the antioxidative potential of peptides derived from various food sources, based on assays that were developed and in common use for measuring antioxidative capacity of non-peptidic antioxidants. Table 6.1 lists most of the research conducted to date in investigating antioxidative potential of protein hydrolysates and peptides derived from various food sources along with the different *in vitro* assay systems utilized for this assessment. Older and very common antioxidative assays include measuring the inhibition of lipid peroxidation in a linoleic acid model system and the capacity to scavenge the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Table 6.1). Kim and co-workers have used the electron spin resonance (ESR) technique to measure the ability of protein hydrolysates and peptides to scavenge DPPH radicals and other reactive oxygen species such as hydroxyl, peroxy, and superoxide as well as carbon-centered radicals (Je and others 2005a,b; Mendis and others 2005; Rajapakse and others 2005; Ranathunga and others 2006; Je and others 2007; Kim and others 2007). Methods such as the Trolox equivalent antioxidative capacity (TEAC) assay and oxygen radical absorbance capacity (ORAC) assay, that have commonly been used in measuring total antioxidative capacity of non-peptidic antioxidants, have also been applied to analyze peptides, but to a lesser extent (Table 6.1).

[1]A version of this chapter will be submitted for publication. Samaranayaka AGP, Li-Chan ECY. Investigation into antioxidative capacity of peptides derived from several food sources, including Pacific hake, using different *in vitro* assay systems.

In the linoleic acid model system, the extent of lipid peroxidation with and without the presence of antioxidant is measured by using the ferric thiocyanate assay (Kikuzaki and Nakatani 1993). Lipid hydroperoxides in the reaction media oxidize Fe^{2+} of the ferrous thiocyanate to Fe^{3+} in order to produce the red colored ferric-thiocyanate complex that can be measured spectrophotometrically at 500 nm. This assay measures the hydrogen atom transfer (HAT) ability of antioxidants to reduce lipid peroxidation (Huang and others 2005), and has been widely used in the past to assess antioxidative capacity of protein hydrolysates and peptides (Table 6.1).

The ORAC assay is an example of another HAT-based assay that applies a competitive reaction scheme where antioxidant and substrate (fluorescein probe) compete for artificially generated hydroxyl radicals (Jimenez-Alvarez and others 2008). As the reaction progresses, the kinetics that describe the decrease of fluorescence due to consumption of fluorescein is retarded by the antioxidant compound present, and is measured with respect to a Trolox calibration curve. Antioxidative capacity is calculated by the difference in the area under the curve (AUC) for blank and the sample (Jimenez-Alvarez and others 2008). The ORAC-fluorescein assay is conducted in an aqueous system and may be applied to assay antioxidant capacity of water-soluble peptides, but does not give an estimation of the antioxidative activity of lipophilic antioxidants (Huang and others 2002). Therefore a modification has been made to this assay using randomly methylated β-cyclodextrin (RMCD) as the molecular host to enhance the solubility of lipophilic antioxidants (Huang and others 2002).

The TEAC and DPPH assays are based on the relative affinity of antioxidant compounds to donate electrons that can scavenge the ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH synthetic radicals, respectively (Huang and others 2005). In the TEAC assay, the pre-formed radical cation is reduced to ABTS in the presence of the antioxidant compound, thus resulting in the loss of color of the reaction solution. The extent of decoloration at 734 nm after 4-8 min of incubation as a function of antioxidant concentration is measured and the TEAC value is obtained by calculating the concentration of antioxidant giving the same percentage inhibition of absorbance of the radical cation as 1 mM of Trolox (Re and others 1999). This assay can be used in measuring antioxidative capacity of both water-soluble and lipid-soluble pure compounds as well as food extracts (Re and others 1999). In the DPPH assay, the purple colour of the DPPH

radical solution is reduced after donation of electrons by the antioxidant compound present in the reaction. A change in colour that can be measured at 517 nm is used to quantify radical scavenging capacity. Since DPPH radical is a relatively stable radical (i.e. easy to work with) as well as a more lipophilic radical (i.e. representative of the radicals formed in the lipid phase), this assay has been widely used in the literature to measure antioxidative capacity of antioxidants. However, for the peptidic antioxidants that are water soluble, use of the DPPH assay that is usually conducted in an 80% ethanol media in measuring antioxidative capacity can be limited due to the reduced accessibility of DPPH radicals by respective amino acid residues in order to donate electrons (Zhu and others 2008). Further, both TEAC and DPPH are end-point assays and therefore the reaction rate differences between antioxidants and oxidants are not reflected in the measurements (Huang and others 2005).

Iron chelating activity is often measured by using a spectrophotometric method whereby the content of free Fe^{2+} ions present after addition of the antioxidant to the reaction mixture is quantified by formation of a colored complex with ferrozine (Decker and Welch 1990).

Although these assays are applied widely to assess activity of non-peptidic antioxidants, the validity of each of these assays to assess the antioxidative potential and underlying mechanism of action of peptides has not been critically evaluated. Further, the effect of primary structural characteristics of peptides that involve both amino acid composition and sequence on related antioxidative capacity is not fully understood. The present study was therefore designed to address these questions using the different *in vitro* antioxidative assay systems mentioned above to characterize the antioxidative potential of five food-derived peptides shown in Table 6.2.

Two of these peptides, Pro-Leu-Phe-Gln-Asp-Lys-Leu-Ala-His-Ala-Lys and Ala-Glu-Ala-Gln-Lys-Gln-Leu-Arg (labeled as A and B, Table 6.2), were identified in a fraction with antioxidative activity that was derived from a Pacific hake fish protein hydrolysate (FPH) made by 1 h autolysis at 52 °C and pH 5.50 (Chapter 4 and Chapter 5). The antioxidative capacity of these individual peptides is unknown.

An antioxidative tripeptide Ala-His-Lys (peptide C, Table 6.2) isolated from egg white albumen (Tsuge and others 1991) shares some homology with the Ala-His-Ala-Lys sequence

found in the C-terminal of the Pacific hake pepide A. Tsuge and others (1991) reported in their study on the tripeptide Ala-His-Lys, that neither the dipeptide sequence His-Lys nor a mixture of the constituent amino acids had any activity, but the dipeptide sequence Ala-His was as potent as the parent tripeptide. In that study, the linoleic acid peroxidation system was used in measuring antioxidative capacity of the protein hydrolysate and isolated peptides (Tsuge and others 1991).

Chen and others (1996) measure antioxidant activities of 28 synthetic peptides designed based on an antioxidative peptide (Leu-Leu-Pro-His-His) derived from a proteolytic digest of soybean protein. This study also used the linoleic acid peroxidation system in assessing antioxidative capacities of these synthetic peptides. The results indicated that removal of C-terminal His residue decreased antioxidative activity, whereas removal of N-terminal Leu had no effect. In the peptide sequence, His and Pro played important roles in the antioxidative activity, and among the peptides tested, Pro-His-His (peptide D, Table 6.2) was the most antioxidative.

The antioxidative peptide Pro-His-His-Ala-Asp-Ser (i.e., peptide E, Table 6.2) identified from tuna cooking juice (Jao and Ko 2002) differs from peptide D by the presence of an additional Ala-Asp-Ser tripeptide sequence at the C-terminal. The DPPH radical scavenging capacity assay was applied to measure antioxidative capacities of the whole hydrolysates from tuna cooking juice as well as semi-purified fractions obtained from Sephadex G-25 and reversed-phase HPLC column separations (Jao and Ko 2002). However, the antioxidative capacities of seven identified peptides, including peptide E (Table 6.2), was not assessed.

The objective of present study was to test the antioxidative potential of chemically synthesized peptides A-E using the linoleic acid peroxidation system, ORAC assay, TEAC assay, DPPH radical scavenging capacity assay, and ferrous ion chelation activity assay. The results obtained by conducting these *in vitro* antioxidant assays are discussed herein with respect to the different reaction conditions, as well as the amino acid composition and sequence of peptides, with the aim of better understanding the different antioxidative mechanisms by which amino acid residues within the peptide sequences may be involved in antioxidative capacity of peptides A-E.

6.2 Materials and Methods

6.2.1 Materials

The five peptides (A-E, Table 6.2) were synthesized by CPC Scientific, Inc. (San Jose, CA) with 90 % purity and kept in sealed vials (5 vials containing 5 mg each, for a total of 25 mg of each peptide) at -80 ºC until used for different antioxidative assays. 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (11557), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (238813), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (D9132), potassium ferricyanide (455946), ferrozine (P9762), ferrous chloride (220299), fluorescein (F6377), 2,2´-azobis-(2-amidinopropane) dihydrochloride (AAPH) (440914), linoleic acid (L1376), and butylated hydroxytoluene (BHT, W218405) were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. Butylated hydroxyanisol (BHA, 101159) was purchased from ICN Biomedicals Inc., Aurora, Ohio. Ethylenediamine tetraacetic acid (EDTA) tetrasodium salt was purchased from VWR International Ltd., Missisuaga, ON.

6.2.2 Antioxidative activity measurements

Inhibition of lipid peroxidation in a linoleic acid model system by synthetic peptides was measured using the method described by Chen and others (1996). BHT and BHA were used as control samples. Peptides were first dissolved in 1.0 mL of 0.1 M PB (final assay concentrations between 0.01-6.0 x 10^{-4} M) in a glass test tube (10 mL volume), followed by the addition of distilled water (0.5 mL) and 50 mM linoleic acid in absolute ethanol (1.0 mL). BHA and BHT control samples were first dissolved in absolute ethanol containing 50 mM linoleic acid (final assay concentrations between 0.01-6.0 x 10^{-4} M), followed by the addition of 0.1 M PB (1.0 mL) and distilled water (0.5 mL). The tubes were sealed tightly and kept at 60 °C in the dark in a shaking incubator. Aliquots (50 µL) of the reaction mixtures were withdrawn after 22 h of incubation for the measurement of antioxidative activity using the ferric thiocyanate method (Mitsuda and others 1966). The reaction mixture (50 µL) was mixed with 75 % ethanol (2.35 mL), 30 % ammonium thiocyanate (50 µL), and 20 mM

ferrous chloride solution in 3.5 % HCl (50 μL). After 3 min, the absorbance of the colored solution was measured at 500 nm. The percent inhibition of lipid peroxidation was measured using the following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

where control is the reaction mixture without peptide samples or synthetic antioxidants. Whole Pacific hake FPH, which was the source of the isolated peptides A and B (see Chapter 4 for details), was also assayed for its antioxidative capacity using the same linoleic acid peroxidation system mentioned above at five different concentrations between 0.001 and 0.2 mg solids/mL in order to compare the results with the results obtained for peptides A and B.

Methods described in Chapter 3 (Section 3.2.7) for the TEAC assay, DPPH assay, ORAC assay, and ferrous ion chelation capacity assay were used to measure antioxidative capacities of synthetic peptides and reference antioxidants. The peptides and Trolox were used at 100 μM and 5 μM final assay concentrations, respectively, for the TEAC assay, and at 150 μM and 15 μM final assay concentrations, respectively, in the DPPH assay. For the ferrous ion chelation capacity assay, peptides and the EDTA reference were prepared at final assay concentrations of 300 μM and 75 μM, respectively. Peptide samples were assayed at several different concentrations for the ORAC assay, as follows: peptides A and E were assayed at five different concentrations between 0.002 and 0.05 mg/mL, peptide C was assayed at five concentrations between 0.01 and 0.25 mg/mL, while peptides B and D were assayed between 0.1 and 1.0 mg/mL.

6.2.3 Statistical analysis

Triplicate samples were used for the ORAC assay and the measurement of lipid peroxidation inhibition in the linoleic acid model system. Duplicate samples were used in performing the DPPH assay, TEAC assay, and the ferrous ion chelation activity assay. Statistical analyses were performed using the SPSS statistical program (SPSS 10.0, SPSS Inc., Chicago, IL). Tukey's test was used to evaluate differences among mean values for treatments at $p < 0.05$.

6.3 Results and Discussion

6.3.1 Antioxidative capacity of FPH peptides in the linoleic acid peroxidation system

Peptides C, D, and E had significantly ($p < 0.05$) higher antioxidative activity in the linoleic acid peroxidation system compared to the peptides A and B derived from Pacific hake FPH (Table 6.3). Tsuge and others (1991) and Chen and others (1996) also reported high inhibitory activity for peptides C and D, respectively in the linoleic acid peroxidation system. At 0.1 and 0.25 $x10^{-4}$ M concentrations, peptides D and E had significantly higher ($p < 0.05$) antioxidant activity than peptide C. The presence of Pro and His in these peptide sequences might have contributed to antioxidative activity (Uchida and Kawakishi 1992; Chen and others 1995).

Although the structure-activity relationship of His-containing peptides with antioxidative activity has not yet been well defined, there is evidence that the activity is attributed to hydrogen donating ability, lipid peroxy radical trapping, and/or the metal ion-chelating ability of the imidazole group (Chan and Decker 1994). The differences in the extent of antioxidant activity of individual His-containing peptides is influenced by the environment surrounding the imidazole group, as indicated by various observations. Murase and others (1993) found that *N*-(longchain-acyl) histidine-containing compounds suppressed the oxidation of phosphatidylcholine liposomes and methyl linoleate. The hydrophobicity of the compounds was important for the accessibility to the hydrophobic targets. Uchida and Kawakishi (1992) investigated the oxidation of Asp-Arg-Val-Tyr-Ile-His-Pro-Phe mediated by copper (II)/ascorbate. The Asp-Arg-Val-Tyr sequence at the N-terminal of the peptide contributed significantly to the reactivity of the His residue, which was converted to the 2-imidazolone derivative upon oxidation. Without the N-terminal segment, the His residue in Ile-His-Pro-Phe showed no reactivity against the oxidation.

The antioxidative activity of peptide E was similar to that of peptide D, indicating that the addition of Ala-Asp-Ser to the C-terminal of the Pro-His-His sequence did not affect the antioxidative potential. At the concentrations tested, BHT had significantly ($p < 0.05$) higher antioxidative activity compared to the peptides and BHA. Antioxidative activity of peptide

A was higher ($p < 0.05$) than that of peptide B at most of the tested concentrations. For both A and B, the antioxidative activity decreased when peptide concentrations reached concentrations higher than 2×10^{-4} M (Table 6.3).

Many antioxidative peptides identified in the literature contain hydrophobic amino acid residues Val, or Leu at the N-terminus of the peptides and Pro, His, or Tyr in the sequences (Uchida and Kawakishi 1992; Chen and others 1995). Peptide A identified from Pacific hake FPH contained Leu in the penultimate position next to the N-terminus, and also contained Pro and His in the sequence. Leu can increase the interaction between peptide and fatty acids (Ranathunga and others 2006). Further, the average hydrophobicity of peptide A (-0.509, calculated as GRAVY value using ExPASy Tools, http://www.expasy.ch/tools/) was higher than that of peptide B (-1.438). These characteristics are potential reasons for explaining the relatively greater antioxidant activity of peptide A in the linoleic acid aqueous system compared to peptide B.

Even though peptide A contained the sequence of Ala-His-Ala-Lys at its C-terminal, which shares some homology to the peptide C (Ala-His-Lys), the antioxidative activity of peptide A was much less than that obtained from peptide C ($p < 0.05$). The presence of different neighbouring amino acid residues to His in the peptide sequence may contribute to steric hindrance or differences in redox potential, which could result in the differences in antioxidant activity noted between the two peptides. For example, in peptide C, the Ala-His residues are known to be very active fragments (Tsuge and others 1991) which has been attributed to the reducing potential that can alter redox potential when in the presence of neighbouring Ala as seen with carnosine (β-Ala-His) (Kohen and others 1988). However, the effect of the presence of Ala residues on both sides of the His residue in peptide A is not known.

Figure 6.1 shows that both peptide A and B had lower antioxidative activity in the linoleic acid peroxidation system than the whole (unfractionated) FPH. One possibility for this finding is the synergistic effects that peptides present in the whole FPH could have in enhancing antioxidative potential compared to the individual peptides. Another possibility is that the presence of higher molecular weight peptides and polypeptides in the FPH could have contributed to the reduction of interfacial tension between the aqueous and oil phases,

and therefore increased the accessibility by lower molecular weight, water soluble antioxidative peptides to the free radicals formed in the non-polar oil phase.

6.3.2 ABTS radical scavenging capacity

The time taken to complete the reaction between ABTS radicals and antioxidative compounds like Trolox, ascorbic acid, and some other phenolic compounds is less than four minutes, as suggested in the improved ABTS radical cation decolorization assay (Re and others 1999). Incubation times of 2-10 min have been employed in previous studies (Nenadis and others 2004; Hernández-Ledesma and others 2005b; Sheih and others 2009).

In the present study, after 8 min incubation, all five peptides possessed marginal ABTS radical cation scavenging activity at 100 μM assay concentration (Figure 6.2). Peptide D had the highest activity followed by peptide A ($p< 0.05$). Addition of the Ala-Asp-Ser tripeptide sequence to the C-terminal of peptide D (i.e., to yield peptide E, Table 6.2) resulted in a decreased reducing capacity. Increasing the incubation time in the assay resulted in different results for the ABTS radical scavenging activity of the peptides and Trolox (Figure 6.2). For Trolox, the % radical scavenging capacity did not significantly change with increasing incubation time up to 30 min (Table 6.4). Peptide D however showed a rapid increase in its ABTS radical scavenging activity with the increased incubation time (Table 6.4), reaching a radical scavenging capacity that was similar to that of Trolox (5 μM) at 60 min (Figure 6.2). The ABTS scavenging activity, as indicated by inhibition of absorbance by peptides A and E, also increased with incubation time (Table 6.4) and peptide E was found to scavenge more radicals than peptide A by 60 min (Figure 6.2). Since the TEAC assay is an end point assay, the present results suggest that longer incubation times are needed for assessing and comparing antioxidative capacity of peptidic antioxidants, compared to other food components, such as polyphenols. Furthermore, this result shows that the peptide sequence greatly affects the ABTS radical scavenging capacity of peptides. Saito and others (2003) reported that 8.2 μM Pro-His-His (i.e., peptide D in this study) had no appreciable activity in the ABTS assay, but the activity was greatly increased by replacing the C-terminal His with Trp or Tyr residues. Aliaga and Lissi (2000) studied the kinetics underlying the mechanism of the reaction between ABTS radical cations and the amino acids Cys, Trp, His, and Tyr.

They found that the relative reactivity followed the order Cys >> Trp > Tyr > His, and that a labile hydrogen atom (e.g., SH, NH, OH) was required for the reaction to occur. This finding explains the reason for the observed increase in ABTS radical scavenging capacity of Pro-His-His, by replacing the C-terminal His with Trp and Tyr (Saito and others 2003). Further, the decrease in radical scavenging capacity in the present study observed when Ala-Asp-Ser was added to the C-terminal of peptide D (i.e. to yield peptide E, Table 6.2) may also be due to the loss of more labile hydrogen at the C-terminal of peptide D. Aliaga and Lissi (2000) proposed a reaction mechanism between these amino acids and ABTS radical cations that involved an initial pH-dependent reversible step, followed by secondary reactions for the substrate-derived radical with itself or with another ABTS radical. In the case with His, they reported a very small, but fast reacting initial response, followed by a slow reaction process with significant autoacceleration at longer times (Aliaga and Lissi 2000). Henriquez and others (2004) also reported a complex reaction mechanism between the ABTS radical cations and polyphenols. They found that the reaction between substrate and ABTS radical occurs in several steps, where the most reactive hydroxyl groups donate hydrogen atoms first, followed by less reactive ones that are present in the polyphenol complex. Therefore, the total reaction in the case of the polyphenolic compounds appears to take a longer time to complete when compared to the reactivity of compounds, such as Trolox, which happens almost instantaneously (Henriquez and others 2004). Further, Henriquez and others (2004) also observed that some secondary reactions of the radical products may lead to partial recovery of ABTS radicals. Even though the mechanism between individual peptides and ABTS radicals has not been studied in detail, the findings from the studies mentioned above may help to explain why marginal reactivity of certain peptides with ABTS radicals occurred at 8 min incubation time, and that longer incubation times were required for peptide D that possesses two His residues at the C-terminal to react with the ABTS radical.

6.3.3 ORAC of peptides

Previous results reported in Chapter 4 indicated significant increases in the oxygen radical absorbance capacity during the peptide purification process from whole Pacific hake FPH. Specifically, ORAC values of 226 ±17, 330 ± 18, 871.2 ± 7.5 and 703.3 ± 36.9 μmol Trolox equivalents/g were determined for FPH, 1-3 kDa UF fraction, and F-10.3 and F-12.3,

respectively (Chapter 4). However, the ORAC value of peptide A was only 99.66 ± 11.67 μmol Trolox equivalents/g and no activity could be observed for peptide B, even when assayed at concentrations up to 1 mg/mL. The possibility of synergistic effects of multiple peptides that could scavenge oxygen radicals might have contributed to the high activity in the semi-purified fraction F12.3. This was the fraction used to obtain peptides A and B. Moreover, a number of characteristics including, functional groups, the amphiphilic nature and solubility of the mixture of peptides present in the semi-purified fraction could have been distinctly different from the characteristics of the single peptides that were chemically synthesized. These differences in composition and related solubility could also be a reason for the reduced activity of synthetic peptides A and B compared to the semi-purified peptide fraction.

Of the five synthetic peptides analyzed during this study, peptide A was shown to possess the highest oxygen radical absorbing capacity ($p < 0.05$) (Table 6.5). The peptide D (Pro-His-His) had no activity up to 2.6 mM assay concentration, but the addition of Ala-Asp-Ser to the C-terminal (i.e. to yield peptide E) resulted in some oxygen radical scavenging capacity (Table 6.5). However, ORAC values of all five peptides were much lower than the values reported for single amino acids such as Trp, Tyr, and Met (4.649, 1.574, and 1.126 μmol Trolox/ μmol amino acid, respectively), as well as for the commonly used synthetic antioxidant BHA (2.43 μmol Trolox/ μmol BHA) (Hernandez-Ledesma and others 2005a). It is of interest that none of these amino acids (Trp, Tyr, Met) were present in the peptide sequences used for this study. Hernandez-Ledesma and others (2005a) further reported that antagonistic effects towards antioxidant activity by some amino acids present in peptide sequences were evident in the oxygen radical scavenging capacity of peptides.

6.3.4 DPPH radical scavenging capacity

Scavenging activity towards DPPH radical was also very low for the peptides tested during this study at 150 μM assay concentration compared to the Trolox reference at 15 μM (Table 6.5). All five peptides were water soluble up to 1 mg/mL according to the information provided by CPC Scientific, Inc., and perhaps the 45 % ethanol media used during this assay may have further compromised solubility factors, as well as their electron donating ability towards the lipophilic DPPH radical (Rival and others 2001b). Peptide A,

with the highest molecular weight and hydrophobicity, possessed the highest ($p < 0.05$) activity. Some literature reports have shown a decrease in DPPH radical scavenging capacity of protein hydrolysates with the extent of protein hydrolysis (Klompong and others 2007; Theodore and others 2008) while others have shown a marked increase in the DPPH radical scavenging activity with the extended protein hydrolysis (Li and others 2007; Raghavan and others 2008). This finding indicates that other than peptide size and solubility, the amino acid composition, sequence, and in the case of protein hydrolyates, abundance of free amino acids (Theodore and others 2008), may also have a key role in determining the DPPH radical scavenging capacity.

Chen and others (1998) reported that Pro-His-His (i.e. peptide D in this study) and other synthetic peptides containing His tested during their study had very low DPPH radical scavenging activity at 33 μM. Further, Leu-Pro-His-His did not have activity even when at 200 μM assay concentration. Rival and others (2001) also reported that synthetic peptides derived from β-casein (Val-Lys-Glu-Ala-Met-Ala-Pro-Lys, Ala-Val-Pro-Tyr-Pro-Gln-Arg, Lys-Val-Leu-Pro-Val-Pro-Gln-Lys, and Val-Leu-Pro-Val-Pro-Gln-Lys) significantly inhibited the AAPH-initiated lipid peroxidation, but could not scavenge DPPH radicals at 100 μM assay concentration. However, other peptides such as glutathione, carnosine, and the peptide Tyr-Phe-Tyr-Pro-Glu-Leu derived from milk casein have been reported to scavenge 50 % of the DPPH radicals in the assay system at concentrations of 6.12, 23.3, and 98 μM, respectively (Suetsuna and others 2000).

Jao and Ko (2002) reported that the semi-purified peptide fraction from tuna cooking juice that contains the peptide E sequence was effective at scavenging 81 % of the DPPH radicals. The solid content of that fraction was 95 μg/mL. However, in the present study, peptide E had very low DPPH radical scavenging capacity even at 150 μM concentration (Table 6.5). It is possible that other peptides in the semi-purified fraction of tuna cooking juice might have contributed, alone or synergistically, in solubilizing peptides and scavenging DPPH radicals.

6.3.5 Ion chelation ability of peptides

The ion chelating ability of peptides A-E according to the method used herein gave poor results compared to the EDTA control (Table 6.5). Furthermore, no significant

differences in relative activities could be observed for the peptides assayed. Rival and others (2001b) also reported that peptides Val-Lys-Glu-Ala-Met-Ala-Pro-Lys, Ala-Val-Pro-Tyr-Pro-Gln-Arg, Lys-Val-Leu-Pro-Val-Pro-Gln-Lys, and Val-Leu-Pro-Val-Pro-Gln-Lys derived from β-casein had very weak Fe^{2+} ion chelating activity even at 500 μM concentration. These researchers also applied the same method as used in the present study and suggested that perhaps the chelating capacity of peptides was underestimated because ferrozine could extract some iron in weaker complexes.

Chen and others (1998) measured Cu^{2+} and Zn^{2+} ion chelating activities of His-containing peptides by monitoring retention times on a immobilized metal affinity column. They observed that generally the retention times increased with the number of His residues present and that peptides with N-terminal His residue showed higher affinity than those with His residues at C-terminus. Pro-His-His (the peptide D in present study) was among the peptides that had long retention time in the Cu^{2+} loaded column. Tsuge and others (1991) also studied the ion chelating activity of egg-white albumen derived peptides of Ala-His-Lys (peptide C in present study), Val-His-His, and Val-His-His-Ala-Asn-Glu-Asn. When a mixture of Fe^{2+} and egg white albumin hydrolysate was separated by Sephadex G-25 gel filtration, most of the bound iron had eluted in the fractions corresponding to the peaks containing antioxidative peptides. These authors therefore suggested that the antioxidative activity of peptides, including Ala-His-Lys, was based on relative iron chelating activity. It is therefore evident that the method use to assess the ion chelating ability of peptides may affect the final result. It should however be noted that even though the ion complexation help in reducing the initiation of oxidative processes in biomolecules, the ion chelating capacity is not always proportional to the antioxidative capacity of individual peptides (Tsuge and others 1991). Nevertheless, results from this assay can be coupled with the results obtained from free radical scavenging capacity assays in order to understand different mechanisms of antioxidative action by peptidic antioxidants.

6.4 Summary and Conclusions

In the present study, peptides A-E displayed different antioxidant activity depending on the *in vitro* chemical assay system used. Molecular characteristics of peptides, at least in part, were responsible for the differences in relative activities as described above. Peptides C, D, and E were more effective in inhibiting the linoleic acid peroxidation, whereas peptide A was more effective in the ORAC assay as well as in scavenging DPPH radicals. Peptide D was the most effective in scavenging ABTS radical cation. Further, the ABTS radical scavenging capacity of peptides D and E showed a asignificant increase with the incubation time, thereby suggesting that the reaction rate may be slower than expected.

According to the literature, having Cys, Trp and Tyr in peptide sequences can contribute to the increase of ABTS radical scavenging potential during the TEAC assay. The presence of Trp and Tyr in the peptide sequence also help increasing the oxygen radical scavenging capacity. Trp and Tyr can act as hydrogen donors due to the presence of indolic and phenolic groups, respectively, and also due to an affinity to form stable indoyl and phenoxyl radicals (Hernandez-Ledesma and others 2005a). Absence of these amino acid residues in the peptides A-E investigated in the present study may have contributed to the low antioxidative potential observed in the TEAC and ORAC assays.

Considering the results obtained from analysis of synthetic peptides A-E using different antioxidative systems in the present study, it was evident that peptides could inhibit the oxidation of linoleic acid more effectively than scavenging radicals, such as ABTS and DPPH. Hydroxyl, peroxy, alkoxy, and carbon-centered radicals are formed during the oxidation of unsaturated fatty acids. The ferric thiocyanate assay used in the present study measures the extent of peroxy radical formation. Jung and others (2007) also reported that the peptide Leu-Val-Gly-Asp-Glu-Gln-Ala-Val-Pro-Ala-Val-Cys-Val-Pro isolated from *in vitro* GI digestion of mussel protein exhibited higher protective activity against polyunsaturated fatty acid peroxidation than ascorbic acid and α-tocopherol. The peptide could further scavenge hydroxyl, superoxide, and carbon-centered radicals exhibiting its potent antioxidative activity towards inhibiting the formation of reactive oxygen species formed by the peroxidation of polyunsaturated fatty acids.

Chen and others (1998) reported that when the peptide Leu-Leu-Pro-His-His was incubated under the conditions used in the linoleic acid peroxidation assay (i.e. in an aqueous ethanol solution with linoleic acid), it was "modified" by a hydroxyl radical. They concluded that the peptide itself functions as a hydroxyl radical scavenger during the process of linoleic acid oxidation. The dipeptide carnosine also scavenges hydroxyl radicals in the linoleic acid assay system (Chan and Decker 1994). Elias and others (2008) reported that highly reactive radicals such as the hydroxyl radical are capable of reacting with the side chains of amino acid residues and therefore are considered to be non-selective scavengers. However, low energy radicals such as hydroperoxyl and DPPH are more selective in abstracting hydrogen atoms first from the most reactive side chains, i.e., Cys, Met, Trp, Tyr, His, and Phe (Elias and others 2008). This distinction in relative radical scavenging activity may explain the differences in the antioxidative capacities observed in different assay systems mentioned above.

As a whole, careful selection and/or validation of antioxidative assays are necessary in measuring and comparing antioxidative capacity of different peptides with antioxidant potentials. Further research is needed in order to elucidate the correlations between molecular characteristics, antioxidative capacity and the mechanism of different peptides.

Table 6.1 Different *in vitro* chemical assays reported in the literature for measuring antioxidative capacity of protein hydrolysates and peptides

Source of peptides or hydrolysates	*In vitro* method(s) used in measuring antioxidative capacity	Reference
Egg white albumin	Linoleic acid peroxidation system	Tsuge and others (1991)
Soybean protein β-conglycinin	Linoleic acid peroxidation system	Chen and others (1995)
Designed peptides based on isolated soy peptide Leu-Leu-Pro-His-His	Linoleic acid peroxidation system	Chen and others (1996)
Capelin whole fish	β-carotene-linoleate model system	Amarowicz and Shahidi (1997)
His-containing peptides designed based on peptides from soy protein digest	Linoleic acid peroxidation system, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide radical scavenging capacity, metal chelating activity, singlet oxygen quenching	Chen and others (1998)
Dried bonito	Methyl linoleate model system	Suetsuna (1999)
Sardine muscle	Superoxide and hydroxyl radical scavenging activity using electron spin resonance (ESR) analysis	Suetsuna and Ukeda (1999)
Casein	Superoxide scavenging activity using tetrazolium salt XTT method, DPPH assay	Suetsuna and others (2000)
Alaska pollack skin gelatin	Linoleic acid peroxidation system	Kim and others (2001)
Lecithin-free egg yolk	Linoleic acid peroxidation system, lipid peroxidation in cultured human liver cells	Park and others (2001)

Source of peptides or hydrolysates	*In vitro* method(s) used in measuring antioxidative capacity	Reference
Casein	Lipoxygenase- and AAPH-catalysed oxidation of linoleic acid; and haemoglobin-catalysed oxidation of linoleic acid hydroperoxide, DPPH assay, Iron chelation ability	Rival and others (2001a,b)
Tuna cooking juice	DPPH radical scavenging capacity	Jao and Ko (2002)
Soybean protein	Liposome-oxidizing system	Pena-Ramos and Xiong (2002)
Wheat gluten	Linoleic acid peroxidation system	Suetsuna and Chen (2002)
Porcine myofibrillar protein	Linoleic acid peroxidation system, DPPH radical scavenging assay, metal ion chelation assay using pyrocatechol violet as the metal chelating indicator	Saiga and others (2003)
Two tripeptide libraries created by combinatorial chemistry	Linoleic acid autoxidation system, Troloxequivalentantioxidative capacity (TEAC) assay, reducing power, peroxynitrite scavenging activity	Saito and others (2003)
Mackerel fillet	DPPH assay, reducing power assay, linoleic acid peroxidation system	Wu and others (2003)
Crude egg white	Oxygen radical absorbance capacity (ORAC) assay, Cu^{2+}-induced low-density lipoprotein oxidation	Davalos and others (2004)
Yellowfin sole frame	Linoleic acid peroxidation system	Jun and others (2004)
Casein phosphopeptides	TEAC assay, DPPH assay, site-specific and non site-specific deoxyribose assays	Chiu and Kitts (2004)
Bovine α-lactalbumin and β-lactoglobulin	ORAC assay	Hernández-Ledesma and others (2005a)
Fermented milk	TEAC assay	Hernández-Ledesma and others (2005b)
Alaska pollack frame protein	Linoleic acid peroxidation system, hydroxyl radical scavenging ability (ESR)	Je and others (2005a)
Hoki frame protein	DPPH, alkyl, hydroxyl-, and superoxide-radical scavenging abilities (ESR)	Je and others (2005b)

Source of peptides or hydrolysates	***In vitro* method(s) used in measuring antioxidative capacity**	**Reference**
Hoki skin gelatin	Carbon-centered, and superoxide radical scavenging activity (ESR), DPPH assay (ESR), Linoleic acid peroxidation system	Mendis and others (2005)
Fermented marine blue mussel	Hydroxyl-, carbon-centered, superoxide-, and DPPH radical scavenging capacity (ESR), metal chelation, linoleic acid peroxidation system	Rajapakse and others (2005)
Chum salmon cartilage and skin	Linoleic acid peroxidation system, DPPH, superoxide, hydroxyl radical scavenging activities	Nagai and Nagashima (2006)
Conger eel muscle	Linoleic acid peroxidation system, hydroxyl-, and carbon-centered radical scavenging activity (ESR)	Ranathunga and others (2006)
Tuna back bone	DPPH, peroxy- and superoxide radical scavenging (ESR), Linoleic acid peroxidation system	Je and others (2007)
Mytilus coruscus muscle protein	Oxidation of polyunsaturated fatty acids, hydroxy-, superoxide-, and carbon-centered radical scavenging activity (ESR)	Jung and others (2007)
Hoki frame protein	DPPH, peroxy-, hydroxyl-, and superoxide radical scavenging (ESR), Linoleic acid peroxidation system	Kim and others (2007)
Yellow stripe trevally	DPPH radical scavenging activity, metal chelation, redcing power	Klompong and others (2007)
Round scad muscle	DPPH radical scavenging capacity, reducing power, Ferrous ion chelation, Linoleic acid peroxidation system, lecithin liposome system	Thiansilakul and others (2007a)
Round scad muscle	DPPH radical scavenging capacity, reducing power, Ferrous ion chelation	Thiansilakul and others (2007b)

Source of peptides or hydrolysates	*In vitro* method(s) used in measuring antioxidative capacity	Reference
Tilapia protein	Isoluminol enhanced chemiluminescent assay in the presence of a) hydrogen peroxide or b) mononuclear cells isolated from human blood. Ferric reducing antioxidant power (FRAP) assay, TEAC assay	Raghavan and others (2008)
Casein, soy protein, and wheat gluten	DPPH assay, AAPH-induced oxidation in the linoleic acid emulsion system	Park and others (2008)
Channel catfish protein isolates	Metal chelating ability, DPPH radical scavenging ability, FRAP, ORAC, and the ability to inhibit the formation of thiobarbituric acid reactive substances (TBARS) in washed tilapia muscle containing tilapia hemolysate	Theodore and others (2008)
Pacific hake	DPPH radical scavenging capacity, ferric ion reducing antioxidative capacity, TEAC assay, ORAC assay, iron chelation, linoleic acid peroxidation system	Samaranayaka and Li-Chan (2008, Chapter 3)
Algae protein waste	TEAC assay, ORAC assay, DPPH-, hydroxyl-, superoxide-radical scavenging capacity, protection against oxidation-induced DNA and cell damage	Sheih and others (2009)

Table 6.2 Synthetic peptides used for the study and their average hydrophobicity values

Synthetic peptide	Molecular mass (Daltons)	Symbol	Origin
Pro-Leu-Phe-Gln-Asp-Lys-Leu-Ala-His-Ala-Lys	1267.5	A	Pacific hake fish fillet[1]
Ala-Glu-Ala-Gln-Lys-Gln-Leu-Arg	943.1	B	Pacific hake fish fillet[1]
Ala-His-Lys	354.4	C	Egg white albumen[2]
Pro-His-His	389.4	D	Soybean protein[3]
Pro-His-His-Ala-Asp-Ser	662.7	E	Tuna cooking juice[4]

[1]Samaranayaka and Li-Chan (2008, Chapter 3) and Chapter 4

[2]Tsuge and others (1991)

[3]Chen and others (1996)

[4]Jao and Ko (2002)

Table 6.3 Antioxidative activity of peptides A-E (sequences shown in Table 6.2), BHA, and BHT in the linoleic acid peroxidation system incubated at 60 °C for 22 h

Sample concen-tration (x 10^{-4} M)	% Inhibition of lipid peroxidation[1]						
	A	**B**	**C**	**D**	**E**	**BHA**	**BHT**
0.01	-0.34 ± 4.96^{b}	-9.69 ± 3.35^{a}	3.44 ± 1.00^{b,c}	8.51 ± 3.78^{c}	-1.42 ± 1.24^{a,b}	ND[2]	ND
0.1	3.96 ± 5.66^{b}	-8.78 ± 5.06^{a}	87.85 ± 1.26^{c}	98.05 ± 0.30^{d}	97.36 ± 0.30^{d}	ND	ND
0.25	1.61 ± 5.78^{b}	-6.81 ± 2.26^{a}	91.95 ± 0.46^{c}	97.97 ± 0.06^{c}	96.70 ± 0.28^{c}	ND	ND
0.5	46.46 ± 12.19^{b}	14.48 ± 13.11^{a}	91.99 ± 0.65^{c}	90.86 ± 0.24^{c}	93.41 ± 2.37^{c}	ND	ND
1.0	55.40 ± 9.37^{b}	18.80 ± 10.93^{a}	90.29 ± 0.32^{c}	91.35 ± 0.32^{c}	90.79 ± 2.66^{c}	ND	ND
2.0	49.79 ± 4.28^{b}	32.13 ± 8.26^{a}	87.67 ± 3.27^{c}	91.85 ± 0.64^{c}	91.35 ± 1.17^{c}	57.16 ± 0.56^{b}	97.16 ± 1.67^{c}
4.0	20.31 ± 7.97^{a}	16.98 ± 6.06^{a}	85.21 ± 0.48^{b}	87.29 ± 0.18^{b}	90.83 ± 3.90^{b,c}	80.83 ± 2.90^{b}	102.08 ± 1.18^{c}
6.0	24.17 ± 0.79^{b}	-1.25 ± 2.56^{a}	86.14 ± 1.18^{c}	91.25 ± 2.25^{c}	87.29 ± 1.30^{c}	91.25 ± 0.83^{c}	102.92 ± 3.43^{d}

[1]Mean values ± Standard deviation from triplicate analyses. Values within each sample concentration (in a row) bearing different letters are significantly different at $p < 0.05$. Values with minus sign indicate prooxidant activity.

[2]Not determined

Table 6.4 Results of statistical analysis of the effect of incubation time on % inhibition of the absorbance of ABTS radical cation by peptides A-E and Trolox[1]

Incubation time (min)	Sample					
	A	**B**	**C**	**D**	**E**	**Trolox**
8	a	a	a	a	a	a
20	a,b	a	a	b	a,b	a
30	b	a,b	a	c	b	a,b
60	c	b	a	d	c	b

[1]Different letters in a column indicate that the % inhibition values are different at $p < 0.05$.

Table 6.5 ORAC, DPPH radical scavenging capacity, and iron chelating ability of peptides A-E[1]

Sample	ORAC value[2]	DPPH radical scavenging capacity (%)[5]	Fe^{2+} ion chelation (%)[6]
A	0.126	4.19[b]	3.36[b]
B	ND[3]	1.98[c]	0.94[b]
C	0.007	1.57[c]	0.86[b]
D	ND	2.27[c]	0.94[b]
E	0.030	2.73[b,c]	1.80[b]
Trolox (15 μM)	NA[4]	34.8[a]	NA
EDTA (75 μM)	NA	NA	84.4[a]

[1]Values shown are mean values of duplicate analysis. Values in a column bearing different letters (except for ORAC values) are significantly different at $p< 0.05$

[2]ORAC = Oxygen radical absorbing capacity (μmol Trolox equi/ μmol peptide)

[3]Activity not detected up to 1.1 mM and 2.6 mM assay concentration for peptides B and C, respectively

[4]Sample not applicable for this assay

[5]DPPH = 1,1-diphenyl-2-picrylhydrazyl. Final concentration of peptides in the assay was 150 μM

[6]Final concentration of peptides in the assay was 300 μM

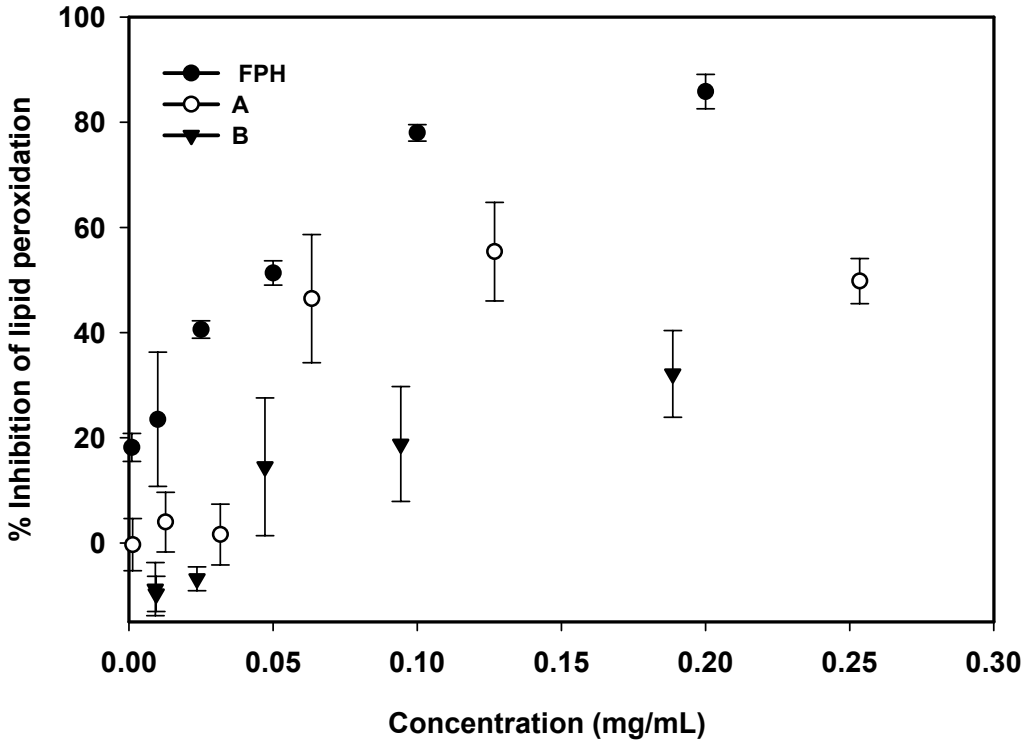

Figure 6.1 Comparison of the antioxidative activity of FPH, peptide A, and peptide B in the linoleic acid peroxidation system

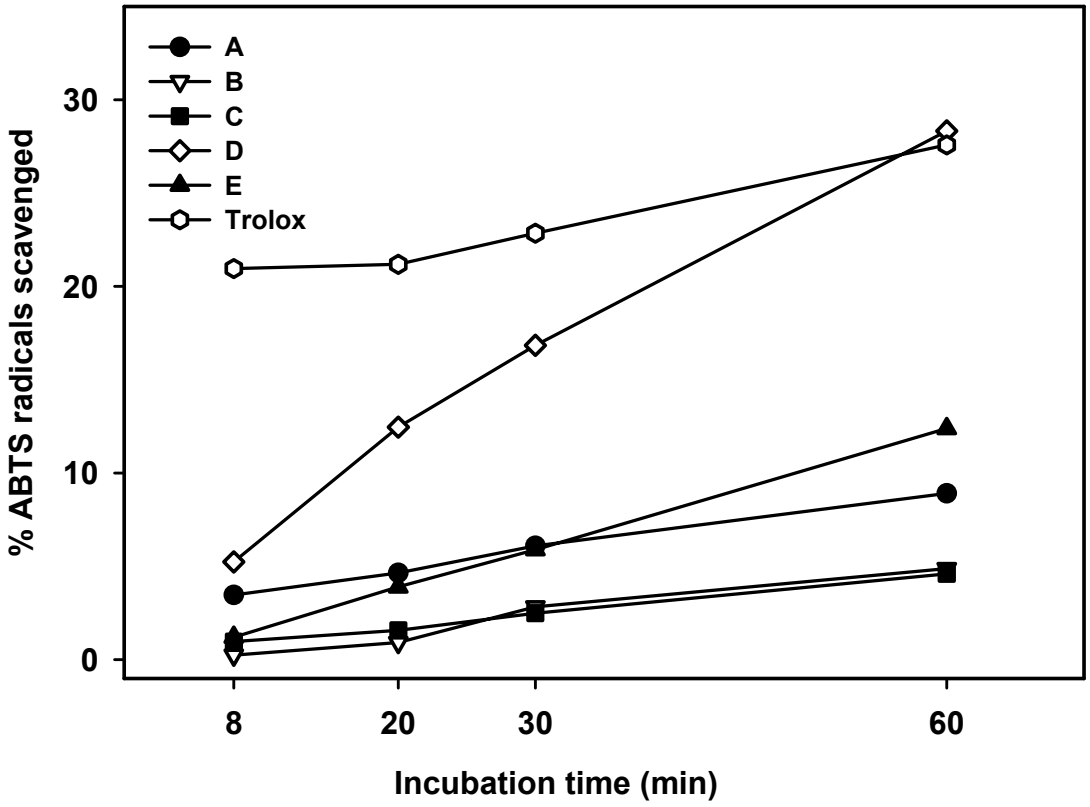

Figure 6.2 ABTS radical scavenging capacity of peptides A-E compared to Trolox

Final concentrations of peptides and Trolox in the assay were 100 μM and 5 μM, respectively. Values shown are mean values of duplicate analysis.

6.5 References

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CHAPTER 7 Conclusion

7.1 Study Findings and Significance

Increased level of endogenous proteolytic activity present in fish muscle of Pacific hake due to *K. paniformis* infection was successfully utilized during the present study to make fish protein hydrolysates (FPH) with antioxidative properties. Production of FPH with antioxidative capacity was demonstrated using several different batches of Pacific hake. It should however be noted that infection level of fish mince should be ~ 10^7 *K. paniformis* spores per gram fish mince or higher in order to obtain FPH with high antioxidative potential through autolysis. In fact, Pacific hake caught near Vancouver Island, BC during summer months are commonly infected with *K. paniformis* at these high levels, according to the data collected by screening of Pacific hake samples in our laboratory during summer and winter months between 2004 and 2006 (unpublished data). However, periodic sampling and assessment of *K. paniformis* infection level is recommended for quality control purposes. Relationships drawn during the present work between *K. paniformis* infection level, endogenous proteolytic activity of fish mince, and free amino acid content during autolysis of fish mince at 52 °C and pH 5.50 will be helpful in selecting the appropriate raw material and controlling processing conditions for antioxidative FPH production. It is also important to control temperature (preferably below 0 ºC) during transportation and handling of fish used for FPH production in order to minimize autolytic processess prior to the controlled autolysis process in making antioxidative FPH.

Alternatively, as shown in Chapter 3, FPH with high antioxidative potential may also be produced from Pacific hake without considering the *Kudoa* infection level by conducting one hour hydrolysis with addition of 2 % commercial enzymes such as Validase® BNP or Flavourzyme® 500L, after first inactivating the endogenous enzymes. Autolysis is more cost-effective since the money spent on enzymes can be saved. Nevertheless, FPH made through 1 h hydrolysis of Pacific hake fish mince using Validase® BNP (2 %) possessed significantly higher ($p < 0.05$) antioxidative capacity towards lipid peroxidation in the linoleic acid model system compared to the FPH made through autolysis (**Appendix IV**). Therefore,

this FPH may also find applications in emulsion-type food systems and potentially *in vivo* in controlling oxidative processess. Further research is however needed to confirm this fact.

FPH made through 1 h autolysis at pH 5.50 and 52 ºC, as well as its 1-3 kDa UF fraction were both effective in controlling lipid peroxidation in the linoleic acid/ethanol/water model system as well as in scavenging oxygen radicals and synthetic radicals such as ABTS. FPH and the 1-3 kDa UF fraction at 0.2 mg/mL concentration showed similar effectiveness in controlling lipid peroxidation (Table 5.1), while the radical scavenging capacity of 1-3 kDa UF fraction evaluated by the TEAC and ORAC assays was higher than that of FPH (Table 4.1). Considering these *in vitro* antioxidative assay results and the need for consistency of the product for commercial purposes, as well as potential organoleptic or quality problems by having other FPH components, it is recommended to use UF for concentrating antioxidative peptides for incorporation into functional food formulations. Further purification of 1-3 kDa UF fraction using ion exchange and size exclusion columns could result in increasing antioxidative potential but the yields of resultant fractions would be quite low for commercial applications.

Potential application of Pacific hake FPH made during the present study and the 1-3 kDa UF fraction as a functional food ingredient to control oxidative processess *in vivo* is further supported by the fact that their antioxidative potential increased upon *in vitro* GI-digestion. These products, when incorporated into functional foods, will therefore have the potential to help reduce intestinal cell injuries and treat a variety of gut conditions similar to the commercial product Seacure®, which is also made from Pacific hake but by yeast fermentation (Fitzgerald and others 2005, Marchbank and others 2008). Further research is however needed to confirm this, since the antioxidative peptides present in Seacure® may be different from those of FPH made through autolysis in the present study.

Experiments conducted with FPH using the Caco-2 cell model system provided evidence to support the possibility that antioxidative peptides permeate the intestinal cell wall and exert antioxidative activity, *in vivo*. Therefore, these antioxidative peptides may have the potential to reach target organs in the body and reduce oxidative damage. Identification of antioxidative peptides however was not complete during the present study and hence the mechanism of antioxidative action as well as the fate of antioxidative peptides during GI-digestion and intestinal transport (i.e. whether degraded by GI enzymes and brush-border

peptidases or absorbed in intact form) are yet to be elucidated. In addition, *in vitro* assays can only predict what may happen *in vivo*, and the true *in vivo* antioxidative efficacy of FPH peptides can only be established by animal studies and subsequent clinical trials.

Even though antioxidative FPH and its GI-digested counterpart possessed *in vitro* ACE-inhibitory capacity, the Caco-2 cell permeate of FPH did not possess any significant ACE-inhibitory activity at the concentrations tested during the present study. It is possible that this FPH may have ACE-inhibitory capacity at higher cellular concentrations, assuming that the peptides exerting ACE-inhibitory activity can pass through the cell membrane. Further, as discussed in Chapter 5, inhibiting ACE activity is not the only mechanism by which peptides can act as antihypertensive agents (Miguel and others 2006). Since this FPH possesses high antioxidative potential, it could contribute to reducing oxidative stress-related damage and thereby reduce hypertension, suggested by other workers (Touyz 2004).

7.2 Recommendations for Future Work

7.2.1 Characterization of antioxidative and ACE-inhibitory peptides present in Pacific hake FPH

Complete identification and characterization of antioxidative peptides from Pacific hake FPH was not successful during the present study. One of the two peptides identified by LC-MS/MS analysis of F-12.3.2 (i.e., Ala-Glu-Ala-Gln-Lys-Gln-Leu-Arg) had very low antioxidative capacity (Chapter 6), while none of the peptides present in F-10.3.5 fraction were identified (Chapter 5). Further, some chromatographic fractions from the Jupiter Proteo 90 Å peptide column separation which had fairly high antioxidative capacity were not further investigated for the peptide identification by LC-MS/MS. Moreover, no attempt was taken to identify ACE-inhibitory peptides from FPH. The identification and characterization of constituent antioxidative and ACE-inhibitory peptides from Pacific hake FPH is therefore necessary in order to elucidate possible antioxidative and ACE-inhibitory mechanisms, as well as to conduct further studies in obtaining required standards of evidence for both antioxidative and ACE-inhibitory potential of bioactive peptides derived from hake FPH before consumer use can be recommended.

The use of semi-preparative or preparative scale columns is recommended to obtain enough sample mass to conduct various assays to confirm the desired bioactivity as well as for further purification and identification purposes. Further, if a large scale column is affordable, it would be more effective to first concentrate antioxidative peptides from FPH using a column such as the Jupiter Proteo 90 Å peptide column, before conducting further purifications. The Jupiter Proteo 90 Å peptide column is a C-12 RP-HPLC column especially designed to separate low molecular weight peptides (<10,000 Da) through hydrophilic/hydrophobic interactions between the analytes and the stationary phase (Phenomenex HPLC Columns for Protein and Peptide Analysis: Jupiter™). In the present study, chromatography of FPH on this column resulted in concentrating most of the antioxidative peptides from FPH into two fractions (i.e. peaks 4 and 6, Figure 4.1 and Table 4.2). The hydrophobic and/or hydrophilic nature characteristic of constituent peptides displaying antioxidant properties might have been the basis for achieving this separation. Further, if antioxidative peptides from FPH can be extracted with sufficient purity, *de novo* sequencing could be a better alternative over the database search used in the present study for identifying the peptides released by autolysis (Contreras and others 2008).

7.2.2 Assay methods and identification of antioxidative mechanisms

The five synthetically made peptides investigated in the present study, including two peptides identified from Pacific hake FPH, had very poor activity towards scavenging synthetic radicals such as DPPH and ABTS, as well as scavenging oxygen radicals during the ORAC assay. It is not clear whether charged groups present in peptides or other structural characteristics hindered access to these radical species. Nevertheless, these peptides could effectively reduce formation of lipid hydroperoxides in the linoleic acid aqueous system. As a whole, it was difficult to explain differences in antioxidative capacities of peptides in different *in vitro* assay systems correlating to their possible mechanisms of action and structural characteristics. Further studies may therefore be needed to elucidate these correlations. One possible approach is making a set of synthetic peptides by altering their structures (e.g. number of positively and negatively charged groups, bulky side chains, hydrophobicity, and sequential elimination and/or inclusion of different amino acids from or to the peptide sequences) in an orderly way so that the differences in antioxidative activities

in different *in vitro* assay systems could be correlated with their structural characteristics and antioxidative mechanisms. Results from this study, with sufficient amount of different peptides being assessed, will also help developing quantitative structure-activity relationship (QSAR) models that can be used in explaining or predicting the structure-activity relationships of other antioxidative peptides.

7.2.3 Further studies to assess *in vivo* efficacy of FPH peptides

As described in Section 1.5.1.3, research conducted in assessing antioxidative potential of peptidic hydrolysates or isolated peptides using animal models or human clinical trials are limited. But these studies are necessary to confirm the results obtained through *in vitro* chemical and cell-based assays. Animal and eventually clinical studies should therefore be conducted with the antioxidative FPH made during the present study.

While promising results have been observed for many of the clinical trials conducted using non-peptidic low molecular weight antioxidants to assess their ability to prevent cardiovascular diseases, different types of cancer, degenerative disorders, and overall mortality, other studies have failed to show any long term beneficial effect (Lotito and Frei 2004; Etminan and others 2005; Pham and Plakogiannis 2005). Koren and others (2008) recently reported that even though there was a dose-dependent increase in the permeability of L-ascorbic acid 2-phosphate in Caco-2, PC3, and Hep3B cell culture systems, no significant increase ($p > 0.05$) in total antioxidative capacity (measured using ORAC, FRAP, and TRAP assays) of the Caco-2 and PC3 cells were observed compared to the control when the cells were challenged with 2′-azobis (2-amidinopropane) dihydrochloride (AAPH) and incubated with ascorbic acid up to 1 mM concentration. Further, a significant decrease in total antioxidative capacity compared to the control was observed in Hep3B cells when incubated with ascorbic acid at 1 mM concentration. Therefore, these authors hypothesized and demonstrated that the total antioxidative capacity of these cell cultures was kept under tight regulation and could not be enhanced by low molecular weight antioxidants. These conflicting results in the scientific literature point to the need for further research to identify the effectiveness of exogenous antioxidants in improving human health as well as the underlying mechanisms involved.

Ability of fish-derived ACE inhibitors to reduce blood pressure is well established through studies conducted with animal models and, more importantly, in human clinical trials conducted with mildly hypertensive subjects (Meisel and others 2006). It is therefore worth re-assessing the *in vivo* ACE-inhibitory capacity of FPH made during the present study. Additional research should also be conducted to assess other possible antihypertensive mechanisms of FPH peptides (Miguel and others 2006).

7.2.4 Incorporation into functional foods

Once the potential biological activities of FPH peptides are established through clinical trials, further research is needed to identify appropriate functional food applications as well as to overcome problems during incorporating the FPH or semi-purified fractions into food matrices.

One possible problem is the bitterness associated with low molecular weight peptides and amino acids present in FPH (Raksakulthai and Haard 2003). During the production of FPH through autolysis of Pacific hake fish filet mince, break down of proteins may mainly be due to the action of cathepsin-L like proteases (An and others 1994). These cysteine proteases prefer to have Arg and Lys at P1 position and Phe, Trp, and Tyr at P2 position during the cleavage (Choe and others 2006). On the other hand, free residues of Arg, Phe, Trp, Pro, Leu, and Ile are found to be bitter (Raksakulthai and Haard 2003). Also, peptides that possess at least two hydrophobic residues at the C-terminal, hydrophobic residues with side chains of at least 3 carbons, and sequences of Arg adjacent to Pro are found to be bitter (Raksakulthai and Haard 2003). Sensory studies will therefore be necessary and if there is any bitterness associated with FPH or 1-3 kDa UF fraction, and possible adjustments to the functional food formulation (i.e. masking), or perhaps, to the antioxidative peptide sequence itself by employing a combination of exo- and endo-peptidases to remove bitter amino acids without loosing intended activity (Raksakulthai and Haard 2003), will be necessary.

Another problem would be the possible interactions of bioactive peptides with other components in the food matrix during processing and subsequent storage. Effect of these interactions on bioactivity of peptides will be an important factor to consider. Encapsulation of peptides is one way to overcome this problem as well as increase delivery of peptides to target sites (Narang and others 2007).

Possible allergic reactions by incorporating bioactive peptides into functional foods will also be a factor to consider (Korhonen and others 1998). FPH and FPH-GI were non-toxic to Caco-2 cells when incubated for 24 h at 0.625 mg/mL concentration (Figure 4.4). Further, FPH and 1-3 kDa UF fraction at concentrations up to 1 mg/mL concentration also showed no toxicity towards HepG2 cells. However, the present study did not look into the possible allergenicity associated with FPH and its semi-purified fractions.

Considering these potential concerns, more research work has yet to be done in creating a functional food using Pacific hake FPH that will both be acceptable by consumers and can successfully deliver these biologically active peptides to target places in the human body without adverse effects.

7.2.5 Food processing applications

As described in Section 1.5.2, protein hydrolysates and peptide mixtures derived from various fish have shown potential to reduce oxidative changes in processed food during storage. Pacific hake FPH made in the present study in fact had higher antioxidative potential than BHA and α-tocopherol in the linoleic acid peroxidation system over prolonged storage (Chapter 3). Even though the possible food applications of this FPH were not studied during the present study, it will be an interesting research area in order to increase utilization of Pacific hake FPH. Further, preliminary studies indicated that this FPH also has ability to reduce surface tension in air/water as well as oil/water interface (**Appendix V**). It would therefore be interesting to assess emulsifying capacity of this antioxidative FPH to explore its potential to act as a multi-functional ingredient in emulsion-type food formulations.

Taken together, Pacific hake, a low-valued fish harvested in huge quantities in BC waters (The British Columbia Seafood Industry Year in Review 2007), is a valuable raw material for the production of multi-functional protein hydrolysates. Especially the promising antioxidative capacity of FPH produced during the present study makes it a potential ingredient to be incorporated into various functional food formulations that target reducing oxidative processes, *in vivo*. With other possible applications of Pacific hake FPH as discussed above, the results from this thesis work will help creating value-added products that will increase utilization and market value of Pacific hake.

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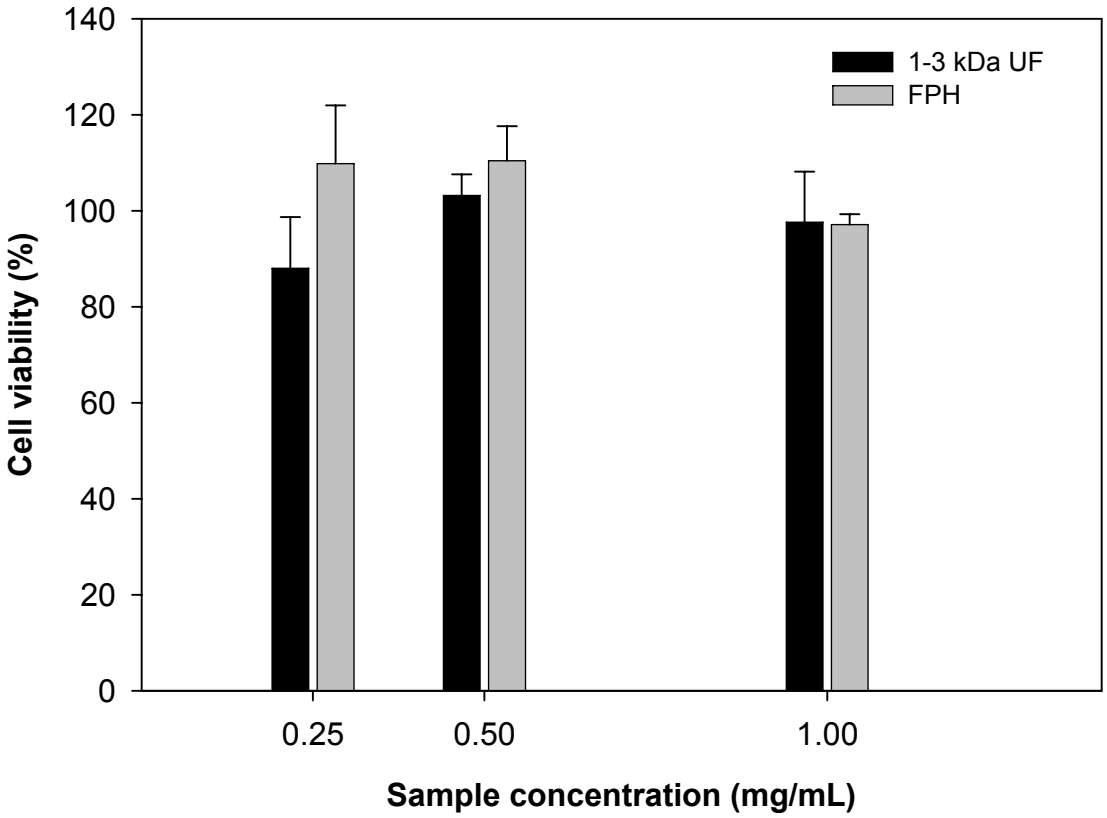
Appendix I Amino acid composition of E-1h and E-6h fish protein hydrolysates (FPH) from Pacific hake

Amino acid	Amount of total amino acids (mg/g sample)	
	E-1h[1]	E-6h[1]
Aspartic acid + Asparagine	134	119
Glutamic acid + Glutamine	244	213
Serine	50	45
Glycine	56	50
Histidine	25	24
Arginine	99	84
Threonine	44	41
Alanine	82	71
Proline	42	41
Tyrosine	37	36
Valine	65	58
Methionine	35	30
Isoleucine	51	50
Leucine	110	93
Phenylalanine	41	37
Lysine	132	119
Cysteine	ND[2]	ND
Tryptophan	ND	ND
Total	604	555

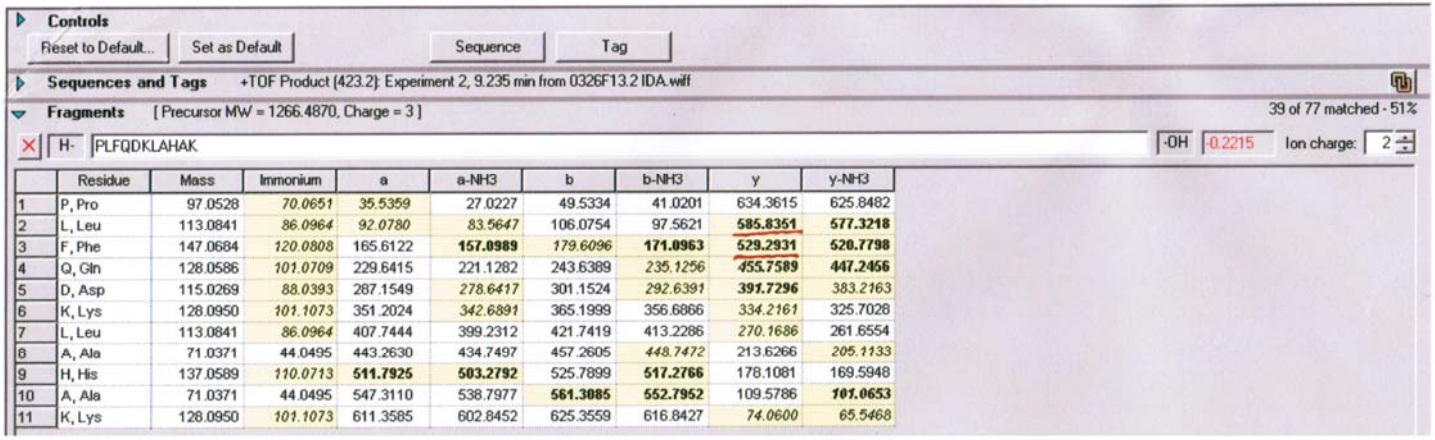
[1]E-1h and E-6h are the FPH made through 1 h and 6 h autolysis, respectively.
[2]Not determined

Appendix II Viability of HepG2 cells after incubation with FPH or 1-3 kDa ultrafiltration fraction at different concentrations



Appendix III Predicted vs matched ions in identifying peptides A (Pro-Leu-Phe-Gln-Asp-Lys-Leu-Ala-His-Ala-Lys) and B (Ala-Glu-Ala-Gln-Lys-Gln-Leu-Arg) using LC-MS/MS

Bold ions are the ones identified from samples by MS/MS analysis



Controls

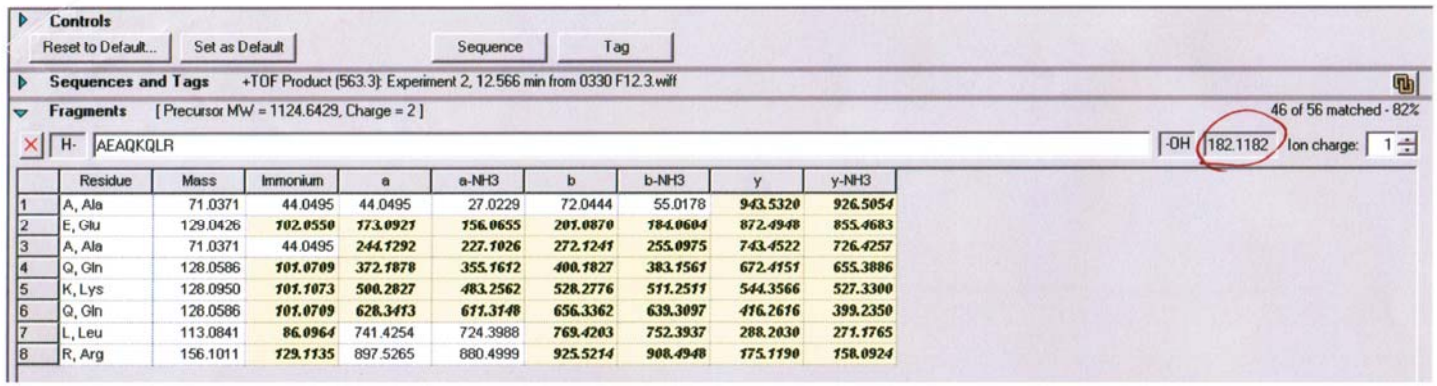
Reset to Default... | Set as Default | Sequence | Tag

Sequences and Tags +TOF Product (423.2): Experiment 2, 9.235 min from 0326F13.2 IDA.wiff

Fragments [Precursor MW = 1266.4870, Charge = 3] 39 of 77 matched - 51%

H- PLFQDKLAHAK -OH -0.2215 Ion charge: 2

	Residue	Mass	Immonium	a	a-NH3	b	b-NH3	y	y-NH3
1	P, Pro	97.0528	*70.0651*	*35.5359*	27.0227	49.5334	41.0201	634.3615	625.8482
2	L, Leu	113.0841	*86.0964*	*92.0780*	*83.5647*	106.0754	97.5621	**585.8351**	**577.3218**
3	F, Phe	147.0684	*120.0808*	165.6122	**157.0989**	*179.6096*	**171.0963**	**529.2931**	**520.7798**
4	Q, Gln	128.0586	*101.0709*	229.6415	221.1282	243.6389	*235.1256*	***455.7589***	***447.2456***
5	D, Asp	115.0269	*88.0393*	287.1549	*278.6417*	301.1524	*292.6391*	***391.7296***	*383.2163*
6	K, Lys	128.0950	*101.1073*	351.2024	*342.6891*	365.1999	356.6866	*334.2161*	325.7028
7	L, Leu	113.0841	*86.0964*	407.7444	399.2312	421.7419	413.2286	*270.1686*	261.6554
8	A, Ala	71.0371	44.0495	443.2630	434.7497	457.2605	*448.7472*	213.6266	*205.1133*
9	H, His	137.0589	*110.0713*	**511.7925**	**503.2792**	525.7899	**517.2766**	178.1081	169.5948
10	A, Ala	71.0371	44.0495	547.3110	538.7977	**561.3085**	**552.7952**	109.5786	***101.0653***
11	K, Lys	128.0950	*101.1073*	611.3585	602.8452	625.3559	616.8427	*74.0600*	*65.5468*



Controls

Reset to Default... | Set as Default | Sequence | Tag

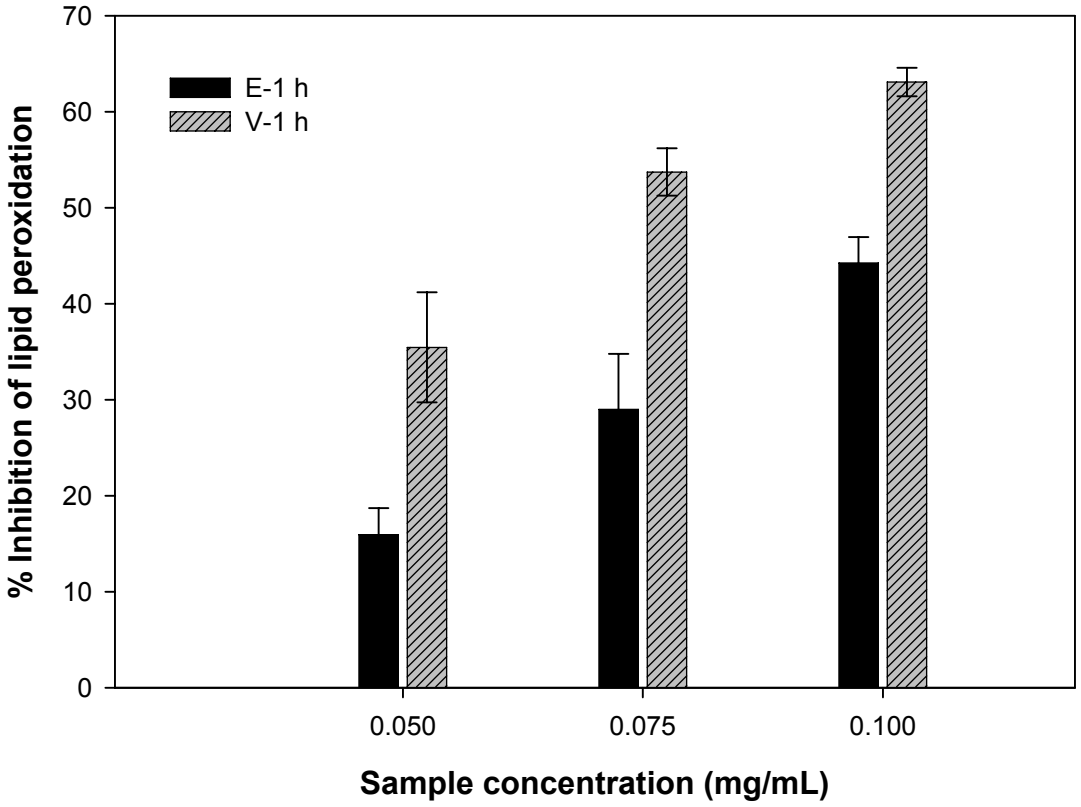
Sequences and Tags +TOF Product (563.3): Experiment 2, 12.566 min from 0330 F12.3.wiff

Fragments [Precursor MW = 1124.6429, Charge = 2] 46 of 56 matched - 82%

H- AEAQKQLR -OH 182.1182 Ion charge: 1

	Residue	Mass	Immonium	a	a-NH3	b	b-NH3	y	y-NH3
1	A, Ala	71.0371	44.0495	44.0495	27.0229	72.0444	55.0178	***943.5320***	***926.5054***
2	E, Glu	129.0426	***102.0550***	***173.0921***	***156.0655***	***201.0870***	***184.0604***	***872.4948***	***855.4683***
3	A, Ala	71.0371	44.0495	***244.1292***	***227.1026***	***272.1241***	***255.0975***	***743.4522***	***726.4257***
4	Q, Gln	128.0586	***101.0709***	***372.1878***	***355.1612***	***400.1827***	***383.1561***	***672.4151***	***655.3886***
5	K, Lys	128.0950	***101.1073***	***500.2827***	***483.2562***	***528.2776***	***511.2511***	***544.3566***	***527.3300***
6	Q, Gln	128.0586	***101.0709***	***628.3413***	***611.3148***	***656.3362***	***639.3097***	***416.2616***	***399.2350***
7	L, Leu	113.0841	***86.0964***	741.4254	724.3988	***769.4203***	***752.3937***	***288.2030***	***271.1765***
8	R, Arg	156.1011	***129.1135***	897.5265	880.4999	***925.5214***	***908.4948***	***175.1190***	***158.0924***

Appendix IV Antioxidative capacity of FPH made through Validase (V-1h) and autolysis (E-1h) in the linoleic acid peroxidation system



Note:

Samples were incubated at 40 °C for 21 h. Values and error bars are the mean and standard deviation from triplicate analyses.

Appendix V Surface tension measurements of FPH and UF fractions

Method

Surface tension of FPH, UF fractions, bovine serum albumin (BSA, A2153 from Sigma), and casein (Casein sodium salt from bovine milk, C8654 from Sigma) were measured using the Surface Tensiomat (Cole-Parmer, model 21, Vernon Hills, IL), and using the duNouy ring method described in the instruction manual of the instrument. Samples were prepared at 0.1, 0.5, and 2.5 mg/mL concentrations in D.D. water. Interfacial tensions between FPH (2.5 mg/mL) and linoleic acid, and BSA (2.5 mg/mL) and linoleic acid were also measured.

Results

Sample	Surface tension[1] at 19 °C (Dynes/cm)
D.D. H_2O	75, 73
FPH	
0.1 mg/mL	67, 66
0.5 mg/mL	63.5, 63
2.5 mg/mL	59, 59
UF fractions at 2.5 mg/mL concentration	
>10 kDa	52, 50.5
3-10 kDa	59, 59
1-3 kDa	62, 61
<1 kDa	63, 63
Bovine serum albumin (BSA)	
0.1 mg/mL	66, 65
0.5 mg/mL	65, 64.5
2.5 mg/mL	59, 57
Casein	
0.1 mg/mL	58, 57.5
0.5 mg/mL	53.5, 53.5
2.5 mg/mL	48.8, 49
	Interfacial tension[1] at 19 °C (Dynes/cm)
D.D. H_2O/Linoleic acid	12, 13
FPH (2.5 mg/mL)/Linoleic acid	4, 5
BSA (2.5 mg/mL)/Linoleic acid	1, 2

[1]Duplicate measurements shown