SEPARATION AND PURIFICATION OF ANTIDIABETIC BIOACTIVE PEPTIDES FROM SALMON AND COD WASTE

by

Tianyi Jin

Submitted in partial fulfillment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
August 2012

© Copyright by Tianyi Jin, 2012

DALHOUSIE UNIVERSITY

DEPARTMENT OF PROCESS ENGINEERING AND APPLIED SCIENCE

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "SEPARATION AND PURIFICATION OF ANTIDIABETIC BIOACTIVE PEPTIDES FROM SALMON AND COD WASTE" by Tianyi Jin in partial fulfillment of the requirements for the degree of Master of Science.

Dated: August 16, 2012

Supervisor: ______________________________

Readers: ______________________________

____

DALHOUSIE UNIVERSITY

DATE: August 16, 2012

AUTHOR: Tianyi Jin

TITLE: SEPARATION AND PURIFICATION OF ANTIDIABETIC BIOACTIVE PEPTIDES FROM SALMON AND COD WASTE

DEPARTMENT OR SCHOOL: Department of Process Engineering and Applied Science

DEGREE: MSc CONVOCATION: October YEAR: 2012

Permission is herewith granted to Dalhousie University to circulate and to have copied for non-commercial purposes, at its discretion, the above title upon the request of individuals or institutions. I understand that my thesis will be electronically available to the public.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

The author attests that permission has been obtained for the use of any copyrighted material appearing in the thesis (other than the brief excerpts requiring only proper acknowledgement in scholarly writing), and that all such use is clearly acknowledged.

____

Signature of Author

TABLE OF CONTENTS

LIST OF TABLES vii

LIST OF FIGURES viii

ABSTRACT........ x

LIST OF ABBREVIATIONS AND SYMBOLS USED........ xi

ACKNOWLEDGEMENTS........ xiii

CHAPTER 1. INTRODUCTION 1

CHAPTER 2. LITERATURE REVIEW........ 3

2.1 TYPE 2 DIABETES........ 3

2.1.1 An Introduction to Type 2 Diabetes 3

2.1.2 Risk Factors for Type 2 Diabetes Development 4

2.1.3 Current Therapeutic Strategies for Type 2 Diabetes 5

2.2 BIOACTIVE PEPTIDES 7

2.2.1 An Introduction to Bioactive Peptides 7

2.2.2 Functionalities of Bioactive Peptides........ 10

2.2.2.1 Common functions of bioactive peptides........ 10

2.2.2.2 Novel functions of bioactive peptides 12

2.2.3 Bioactive Peptides from Marine Fish Waste........ 13

2.3 CURRENT STATUS OF UTILIZATION OF FISH WASTE 16

2.4 METHODOLOGY 17

2.4.1 Production of Protein Hydrolysates........ 17

2.4.1.1 Chemical methods 17

2.4.1.2 Enzymatic hydrolysis 19

2.4.2 Separation and Purification of Bioactive Peptides........ 22

2.4.3 Identification of Bioactive Peptides........ 28

CHAPTER 3. OBJECTIVES 29

CHAPTER 4. MATERIALS AND METHODS 30

4.1 SAMPLE PREPARATION 30

4.2 LAB SCALE ENZYMATIC DIGESTION OF SALMON AND COD 30

MUSCLE PROTEINS...30

4.2.1 Protocol 1 (0.1 M NaOH dissolution; pepsin / pancreatin digestion)..............32

4.2.2 Protocol 2 (1.0 M NaOH dissolution; pepsin, trypsin and chymotrypsin digestion)...33

4.2.3 Protocol 3 (Water dispersion; pepsin, trypsin and chymotrypsin digestion) 33

4.3 PROCESS SCALE UP 32

4.3.1 Two hundred and fifty g batch 34

4.3.2 Two thousand five hundred g batch 35

4.3.3 Twelve and one half kg batch 36

4.4 TESTING REPRODUCIBILITY OF PEPTIDE PRODUCTION 37

4.5 DETERMINATION OF NITROGEN CONTENT AND RECOVERY 38

4.6 EFFECT OF ENZYME CONCENTRATION ON NITROGEN RECOVERY FOR SALMON DIGESTS (PROTOCOL 2) 38

4.7 OPTIMIZATION OF INCUBATION TIME FOR SALMON DIGESTION (PROTOCOL 2) 40

4.8 DETERMINATION OF PEPTIDE PROFILES OF THE FISH PROTEIN HYDROLYSATES 41

4.9 GEL FILTRATION OF SALMON AND COD PROTEIN HYDROLYSATES 42

4.10 PREPARATIVE HPLC FRACTIONATION OF SALMON AND COD PROTEIN HYDROLYSATES 42

4.11 ION EXCHANGE CHROMATOGRAPHY 43

4.12 FURTHER HPLC ANALYSIS..44

CHAPTER 5. RESULTS AND DISCUSSION 45

5.1 PRODUCTION OF FISH PROTEIN HYDROLYSATE..45

5.1.1 Mechanical deboning of fish frames...45

5.1.2 Nitrogen recovery 46
5.1.3 Reproducibility of the scaled-up process...50
5.1.4 The effect of enzyme concentration on nitrogen recovery 52
5.1.5 Optimization of incubation time 55
5.2 CHROMATOGRAPHIC SEPARATION OF PEPTIDES FROM FISH PROTEIN HYDROLYSATES 58
5.2.1 Peptide profile of the fish protein hydrolysates (FPH) 59
5.2.2 Size-exclusion chromatography (Gel filtration) 62
5.2.3 Preparative-scale HPLC fractionation of salmon and cod protein hydrolysates 66
5.2.4 Ion exchange chromatography..69
5.3 BIOACTIVITY OF THE FISH PROTEIN HYDROLYSATE 71
5.3.1 Bioactivity of original eight fish protein hydrolysates (<1 kDa).....................71
5.3.2 Bioactivity of selected two fish protein hydrolysates (<1 kDa) 71
5.3.2.1 Glucose production assay 72
5.3.2.2 Inflammation modulation 72
5.3.2.3 Adiponectin metabolism modulation...72
5.3.2.4 *In vivo* mouse feeding trials..73
5.3.3 Bioactivity of peptide fractions from preparative HPLC fractionation 73
5.3.3.1 Bioactivity of fish protein hydrolysates (<1 kDa) fractions separated on a Jupiter C_{12} column 73
5.3.3.2 Bioactivity of Superdex Peptide 10/300 GL column separated fractions of fish protein hydrolysates (<1 kDa)...74
CHAPTER 6. CONCLUSIONS 75
6.1 CONCLUSIONS 75
6.2 FUTURE WORK 76
REFERENCES 77

LIST OF TABLES

		Page
Table 2.1	Current dietary recommendations for people with diabetes……..	6
Table 2.2	Commercially available marine protein hydrolysate and peptide products ………………………………………………………	9
Table 2.3	Peptides with anticancer activities.……………………………...	12
Table 2.4	Marine fish waste derived protein hydrolysates / peptides.……...	15
Table 2.5	The cleavage specificities of common proteolytic enzymes.…….	21
Table 2.6	Optimum reaction conditions of common proteolytic enzymes…	21
Table 2.7	Common methods for separation of peptide mixtures…………...	22
Table 4.1	The elution gradient for HPLC of peptides………………………	38
Table 4.2	Enzyme concentrations used for determining effect on nitrogen recovery ………………………………………………………	39
Table 4.3	Elution gradients for preparative HPLC fractionation of fish protein hydrolysates……………………………………………...	43
Table 5.1	Deboning yields……………………………………………….	45
Table 5.2	Yield of each production step and the nitrogen recovery of fish protein hydrolysate..	49
Table 5.3	The 2012 price of enzymes used in this study…………………...	53
Table 5.4	Nitrogen recoveries of salmon using different enzyme concentrations (pepsin/trypsin/chymotrypsin)…………………...	53

LIST OF FIGURES

		Page
Figure 2.1	Impact of type 2 diabetes……………………………………….….....	4
Figure 2.2	A flow chart of the typical production of protein hydrolysates…...	18
Figure 2.3	The principle of acid hydrolysis of protein………………………	19
Figure 2.4	The principle of gel filtration……………………………………..	24
Figure 2.5	The principle of ion exchange chromatography…………………..	26
Figure 4.1	Protocols for producing salmon and cod protein hydrolysates …..………………………………………………...	31
Figure 4.2	Schematic diagram of the TFF filtration apparatus……………….	35
Figure 4.3	Protocol for determining the effect of different enzyme concentrations on nitrogen recovery...	40
Figure 5.1	RP-HPLC peptide profiles of different batches of salmon protein hydrolysate (<1 kDa)………………………….........................	51
Figure 5.2	HPLC chromatograms of enzymatic hydrolysates of salmon mince with different enzyme concentrations.................................	54
Figure 5.3	Effect of pepsin concentration on salmon protein hydrolysis…….	56
Figure 5.4	Effect of trypsin/chymotrypsin concentration on the hydrolysis of peptic digests of salmon……………………....…………..............	57
Figure 5.5	RP-HPLC peptide profile of the salmon protein hydrolysate (<1 kDa)…………………………..	60
Figure 5.6	RP-HPLC peptide profile of the cod protein hydrolysate (<1 kDa)……………………..	61
Figure 5.7	Elution profiles of the standards on the Superdex Peptide 10/300 GL column and the relationship between log MW and V_e of standards..	63
Figure 5.8	Gel filtration of the salmon protein hydrolysate (<1 kDa) on a Superdex Peptide 10/300 GL column……………………….…….	64
Figure 5.9	Gel filtration of the cod protein hydrolysate (<1 kDa) on a Superdex Peptide 10/300 GL column...	65
Figure 5.10	Preparative HPLC fractionation of salmon protein hydrolysates on a Jupiter C_{12} column..	67

Figure 5.11 Preparative HPLC fractionation of cod protein hydrolysates on a Jupiter C_{12} column.. 68
Figure 5.12 Ion exchange chromatography of salmon protein hydrolysate on UNO sphere Q column…………………………………………… 70

ABSTRACT

Dietary proteins from Atlantic salmon and cod have previously been shown to have antidiabetic effects. Since dietary proteins are digested into small peptides before being absorbed through the intestinal mucosa, it is reasonable to deduce that the antidiabetic effect is due to enzymatically-digested peptides rather than the proteins themselves. The aim of this study was to develop a protocol to recover peptides with antidiabetic effects from salmon and cod protein digests and then scale up and optimize the salmon protein hydrolysate production process for industrial-scale production. The peptide mixtures were screened using cell culture assays for insulin-modulating activities and were further fractionated and purified for the final identification. Total yields of salmon and cod protein hydrolysates (<1 kDa) as measured by Kjeldahl nitrogen were 16.9% and 40.1%, respectively. The production process used for the salmon protein hydrolysate (<1 kDa) showed good reproducibility and potential for the industrial-scale production.

LIST OF ABBREVIATIONS AND SYMBOLS USED

T2D	Type 2 diabetes
EASD	European Association for Study of Diabetes
ADA	American Diabetes Association
BMI	Body mass index
ACE	Angiotensin-I-converting enzyme
MW	Molecular weight
LSEL	Leu-Ser-Glu-Leu
LMW	Low molecular weight
HIC	Hydrophobic interaction chromatography
RP-HPLC	Reverse-phase high-performance liquid chromatography
TFA	Trifluoroacetic acid
MS	Mass spectrometry
FPH	Fish protein hydrolysate
NaOH	Sodium hydroxide
dH_2O	Distilled water
E:S	Enzyme : substrate
TFF	Tangential Flow Filtration
PDA	Photodiode array
NR	Nitrogen recovery
TCA	Trichloroacetic acid
V_e	Elution volume
V_o	Void volume
AN	Acetonitrile
MW_{app}	Apparent molecular weight
HGP	Hepatic glucose production

SD	Standard deviation
SEM	Standard error of the mean
NO	Nitric oxide
MWCO	Molecular weight cut off

ACKNOWLEDGEMENTS

This thesis would not have been possible without the guidance and the help of several individuals. First and foremost I offer my sincerest gratitude to my supervisor, Dr. Tom Gill, who has supported me throughout this research with his patience, knowledge, encouragement and funding, and without him this thesis would not have been completed or written.

In addition, I am especially grateful to Dr. Fida Hasan who has provided me lots of assistance and advice in my M.Sc. pursuit. My thanks and appreciation also goes to John Thompson and Doug Singer for their help with all the technical problems; our partners — Dr Marette's laboratory at Laval University and Dr McLeod's laboratory at Dalhousie University for their cooperation, and a huge "thank you" to all my lab buddies.

Finally, thanks to my parents and friends for supporting me throughout all my studies at university.

CHAPTER 1. INTRODUCTION

The incidence of type 2 diabetes (T2D) is rising dramatically all over the world. According to the International Diabetes Federation (2010) report, 285 million people are affected by T2D worldwide (Jain & Saraf, 2008). In Canada, approximately 2.7 million adults are suffering from this disease and this number is expected to be 3.3 million by 2020 (Canadian Diabetes Association, 2010). Usually, T2D occurred in people over the age of 40. However, in the last two decades, T2D has been becoming more and more common in younger people, driven by an “obesogenic” environment (Fox & Kilvert, 2007). A number of therapeutic strategies have been developed for T2D, but its progressive nature and considerable therapeutic expenses require research toward more effective and economical treatments.

A group of researchers at the Faculty of Medicine at Laval University reported that when cod and salmon proteins were used as sole dietary protein sources in animals prone to T2D, a significant increase in insulin sensitivity was observed (Lavigne et al., 2001; Pilon et al., 2010). In their subsequent human experiments, dietary cod protein also showed the ability to improve insulin sensitivity in insulin-resistant individuals (Ouellet, 2007). T2D is manifested by insulin resistance, so these results demonstrated the potential for dietary cod and salmon protein as a new alternative to treating T2D. Since dietary proteins are digested into small peptides or amino acids prior to being absorbed through the intestinal mucosa, it is reasonable to assume that the bioactive effects of these fish proteins are due to enzymatically-degraded peptides rather than the proteins themselves. Indeed, in many cases, bioactive peptides display higher bioactivities than the intact proteins from which they are derived (Van Regenmortel, 1999; Hosomi et al., 2010). Therefore, the present study was initiated to isolate and identify insulin-modulating peptides from cod and salmon proteins. Cod and salmon proteins can be recovered from industrial fish processing waste. According to the Canada Aquaculture Statistics Report (2009), in 2009, the total production of salmon

in Canada was 9.09×10^7 kg. Generally, only the fillet is retained for human consumption; nearly 60% of the fish is discarded or only used for producing silage or fish meal which has relatively small economic benefit. Actually, a large part of the filleting waste products could be transformed into high-value products like enzymes, gelatine and bioactive peptides (Arvanitoyannis & Kassaveti, 2008). The utilization of fish waste not only increases the value of by-products, but also improves the profit margins for the aquaculture industry while diverting organic material from the waste stream, thus helping to solve the environmental problems caused by the waste.

CHAPTER 2. LITERATURE REVIEW

2.1 TYPE 2 DIABETES

2.1.1 An Introduction to Type 2 Diabetes

Type 2 diabetes was first identified in the 19th century. According to the Diabetes Dictionary of the Canadian Diabetes Association, type 2 diabetes is "a condition in which the body either cannot produce enough insulin or cannot effectively use the insulin it produces" (Rosenthal, 2007). Actually, both of these two symptoms appear in the diabetic individual. In the early stage of this disease, type 2 diabetics produce normal or even elevated levels of insulin but the body becomes insensitive to this essential hormone, and the condition is referred to as insulin resistance. In advanced stages, the predominant abnormality is the reduced insulin secretion by the pancreatic β-cells (DeFronzo, 1997; Reaven, 1988; Virally, 2007).

Type 2 diabetes is a chronic disease which may develop undetected for many years (Fox & Kilvert, 2007). Patients with type 2 diabetes may have symptoms such as weight gain, blurred vision, drowsiness, tingling or numbness in hands and feet, and gum disease (Rosenthal, 2007). Most people with type 2 diabetes have no obvious symptoms for a long time. This doesn't mean type 2 diabetes is a mild disease. Indeed, it threatens human health and is often manifested with serious complications caused by high glucose levels in the blood (Figure 2.1). Some of these complications can even result in death (Metzger, 2006).

In the past three decades, the morbidity of type 2 diabetes has sharply increased due to the rapid socio-economic development and dramatic changes in lifestyle. This phenomenon was more obvious in developing countries than developed countries (Haffner, 1998). Another significant change in recent years is the increasing incidence of type 2 diabetes in children and adolescents. Researchers have even predicted by 2019, type 2 diabetes would

be the major form of diabetes in younger people instead of type 1 diabetes within 10 years (Serrano, 2009).

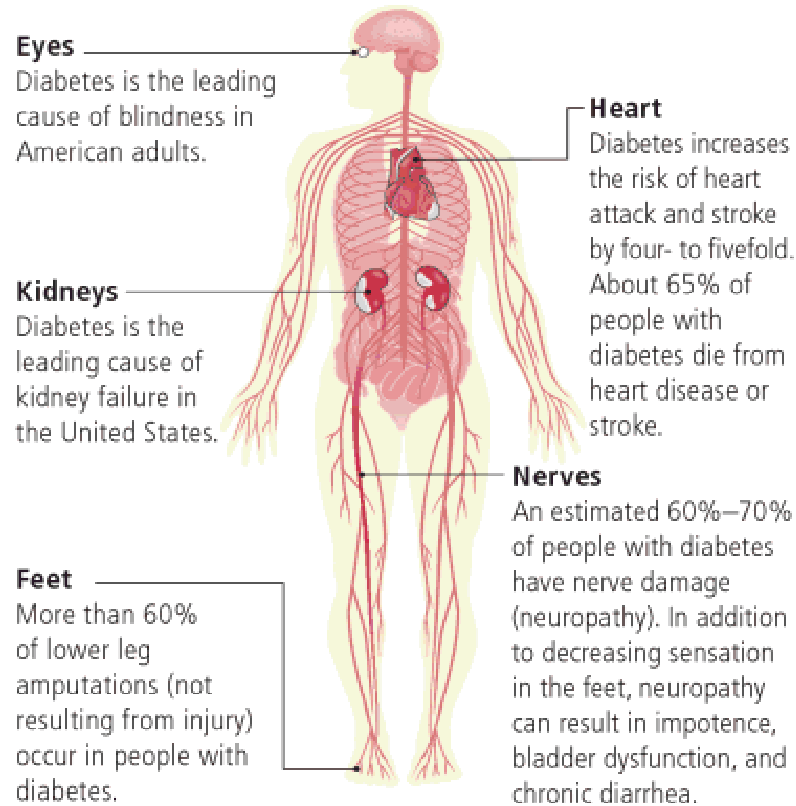

Figure 2.1 Impacts of type 2 diabetes (Stephanie, 2009, with permission)

2.1.2 Risk Factors for Type 2 Diabetes Development

Type 2 diabetes is often named as "lifestyle disease" since it is largely due to obesity, lack of exercise and poor diet. It is reported that 80 to 90 percent of type 2 diabetics are overweight or obese (Cabot & Jasinska, 2007). Therefore, the most critical risk factor for type 2 diabetes is obesity. Generally, the longer you have been obese, the more you are at risk because obesity can induce insulin resistance, which in turn, causes type 2 diabetes (Kahn et al., 2006). Besides obesity, numbers of other factors also affect the development of type 2 diabetes. Rosenthal (1999) divided these factors into two groups: "modifiable risk factors" and "non-modifiable risk factors". Modifiable risk factors are the factors that can be changed by humans, like obesity, high cholesterol, hypertension, sedentary lifestyle,

smoking, sleep deprivation or sleep disorders and vitamin deficiency. Non-modifiable risk factors refer to the factors cannot be changed such as age, genetics, gender, race and medication.

2.1.3 Current Therapeutic Strategies for Type 2 Diabetes

Type 2 diabetes is also called non-insulin-dependent diabetes, since people who have type 2 diabetes do not rely on daily insulin injection at least in the early stages of the disease (Peacock, 2000). However, as the disease develops, patients gradually acquire a dependence on insulin therapy. Insulin therapy is perhaps the most effective treatment which can increase insulin secretion by reducing hyperglycemia and improving the sensitivity to insulin. Sometimes it can even reverse insulin resistance (Campbell & White, 2002). Insulin therapy is often used in the later stages of type 2 diabetes when other therapeutic strategies are no longer effective. However some diabetic experts pointed out that starting insulin therapy in the early stage of type 2 diabetes could help preserve remaining β-cell function and improve long-term glycemic control (Glaser & Cerasi, 1999).

In addition to insulin therapy, there are a lot of other treatments for type 2 diabetes. In its early stage, type 2 diabetes can be controlled by lifestyle changes which include healthy diet and exercise. Dietary therapy plays a key role in preventing and managing type 2 diabetes (Orozco et al., 2008). Professional dietary suggestions for type 2 diabetes have been established in Europe, USA and Canada (Choudhary, 2004). The current suggestions from the European Association for Study of Diabetes (EASD) and the American Diabetes Association (ADA) are listed in Table 2.1.

Exercise therapy is also important in achieving good glycemic control. Colberge (2006) stated that type 2 diabetes could be prevented and controlled with increased physical activity. Fritz et al. (2005) reported that increasing regular physical activity, like 45 minutes of walking 3 days per week can help enhance systolic and diastolic blood pressure, promote lipid metabolism, and decrease body mass index (BMI).

Although proper diet and physical exercise can help improve the condition of type 2

diabetes, patients are often reluctant to change their original lifestyle. On the other hand, the disease has a progressive nature. Exercise and proper diet may not completely solve the problem. Therefore, pharmacological intervention becomes necessary to treat type 2 diabetes. Traditional medication includes various oral hypoglycemic agents such as sulfonylurea, biguanides, α-glucosidase inhibitors and thiazolidinediones (Bailey, 2000). Different medications have different action mechanisms. Doctors can choose the most suitable one for patients according to their disease status (Jain & Saraf, 2008).

Table 2.1 Current dietary recommendations for people with diabetes (Choudhary, 2004).

COMPONENT	European Association for Study of Diabetes (EASD)	American Diabetes Association (ADA)
Carbohydrate + monounsaturated fatty acids (% of daily energy)	60–70%	60–70%
As sucrose (% of daily energy)	10%	10%
Fat (% of daily energy)	Total fat intake ≤35%	Total fat intake ≤35%
Saturated fatty acids (% of daily energy)	<8–10%	<7–10%
Polyunsaturated fatty acids (% of daily energy)	<10%	~10%
Protein (% of daily energy)	10–20%	15–20%
Fiber	Intake encouraged	Intake encouraged
Salt	<6 g/day	<6 g/day

These treatments can be used alone or in combination to enhance the effect for treating type 2 diabetes. Unfortunately, none of the therapeutics can completely cure this disease and some even have side effects. For example, insulin therapy may cause hypoglycemia, weight gain, increase adiposity and aggravate insulin resistance (Bailey, 2000). Dietary therapies are highly individualized since different patients have different situations. Exercise therapy has been used to treat the disease but the synergy between insulin action and physical

activity was not clear until recently; patients' special metabolic situation may lead them into hypoglycemic or hyperglycemic conditions (Colberge, 2006).

In recent years, the pathogenic mechanisms of type 2 diabetes have become clearer which has led to the development of novel treatment strategies, such as the detection of new potential drug targets (Moller, 2001) and the development of incretin-based therapies which act via certain pathway that affect glucose metabolism (Campbell, 2002). However, most of these recently-developed strategies are still in preclinical or early clinical stages. Their effectiveness and potential side effects need to be further studied. There is still an urgent need for new effective and safe antidiabetic agents.

2.2 BIOACTIVE PEPTIDES

2.2.1 An Introduction to Bioactive Peptides

As the name implies, bioactive peptides refer to peptides which have biological activity and may influence human health (Kitts & Weiler, 2003). These peptides are often inactive or less active within the sequence of their parent protein and need to be released by digestion or hydrolyzing protein *in vitro* before becoming active (Erdmann et al., 2007). Bioactive peptides widely exist in natural sources such as milk, eggs, plants and animals (Hartmann & Meisel, 2007). Besides being produced by gastrointestinal digestion or by hydrolyzing protein *in vitro*, bioactive peptides can also be synthesized by chemical methods or DNA recombinant technology (Li, 2007).

Usually, bioactive peptides are small and consist of 2-20 amino acids (Daffre et al., 2008). However, there are a few exceptions. Lunasin, for example is a peptide with anticancer activity and contains 43 amino acids (Jeong et al., 2002). The number and sequence of

amino acids are the most important factors affecting the activities of bioactive peptides studied to date.

Nowadays, research on bioactive peptides is a hot topic because of their high absorption efficiency and unique physiologic functions (Shi & Chen, 2000). Bioactive peptides have been identified with a wide variety of functions which have various applications in food, cosmetic and pharmaceutical industries. Currently, soy and dairy peptides are dominating the bioactive peptide market while marine fish-derived peptides are not as significant. Table 2.2 lists some currently available commercial products containing marine fish protein hydrolysates/peptides as functional ingredients. Among these, Peptide ACE 3000 and Lapis support have been approved by Japanese authorities (Thorkelsson & Kristinsson, 2009).

Table 2.2 Commercially available marine protein hydrolysate and peptide products (Harnedy & Fitzgerald, 2011)

Product	Activity	Source	Manufacturer
PeptACE™	Antihypertensive	Bonito peptides	Natural Factors Nutritional Products Ltd., Canada
Vasotensin®	Antihypertensive	Bonito peptides	Metagenics, US
Levenorm®	Antihypertensive	Bonito peptides	Ocean Nutrition Canada Ltd.
Peptide ACE 3000	Antihypertensive	Bonito peptides	Nippon Supplement Inc., Japan
Lapis Support	Antihypertensive	Sardine peptides	Tokiwa Yakuhin Co. Ltd., Japan
Valtyron®	Antihypertensive	Sardine peptides	Senmi Ekisu Co. Ltd., Japan
Stabilium® 200	Reducing stress	Fish autolysate	Yalacta, France
Protizen®	Relaxing	Fish hydrolysate	Copalis Sea Solutions, France
AntiStress 24	Reducing stress	Fish hydrolysate	Forté Pharma Laboratories, France
Nutripeptin™	Lowers glycemic index	cod hydrolysate	Nutrimarine Life Science AS, Norway
Seacure®	Strengthen intestinal integrity	Pacific whiting hydrolysate	Proper Nutrition, US
Fortidium Liquamen®	Antioxidant, lowers glycemic index, anti-stress	Fish autolysate	Biothalassol, France

2.2.2 Functionalities of Bioactive Peptides

Bioactive peptides have numerous functions. Perhaps the most common are antihypertensive, antioxidant, immunomodulatory and antimicrobial activities (Korhonen & Pihlanto, 2005). Besides the common ones, several novel functions of bioactive peptides have been discovered, such as anticancer, anti-obesity and antidiabetic activities (Kim & Mendis, 2006; Shahidi & Zhong, 2008; Erdman et al., 2008). Moreover, some peptides may exhibit multifunctional properties (Meisel, 2004). Details of these functions are given below.

2.2.2.1 Common functions of bioactive peptides

Antihypertensive activity is probably the most extensively studied function of bioactive peptides (Shahidi & Zhong, 2008). Antihypertensive peptides can inhibit the activity of the angiotensin-I-converting enzyme (ACE) which is closely linked with high blood pressure (Erdmann, 2007). Numerous sources like milk, eggs, fish and soy, have been used for extracting peptides with antihypertensive activity. Scientists found that some of the antihypertensive peptides have a commonality in structure. Most of the antihypertensive peptides are short sequences containing from 2 to 12 amino acids (Mine & Shahidi, 2006). In addition, the three C-terminal amino acids of antihypertensive peptides are usually hydrophobic residues (Vermeirssen et al., 2004), since the ACE prefers substrates or inhibitors containing hydrophobic amino acid residues at each of the three C-terminal positions (Ledesma et al., 2010).

Antioxidant peptides can reduce the peroxidation of lipids or fatty acids, scavenge free radicals and chelate transition metal ions (Moure et al., 2006; Qian et al., 2008; Rajapakse et al., 2005). Numerous antioxidant peptides have been identified from diverse animal and plant sources (Sarmadi & Ismail, 2010). Among the antioxidant peptides, the marine-based are perhaps the most important. Several studies suggested that peptides derived from

marine fish proteins may have higher antioxidant activity than α-tocopherol (a form of Vitamin E) (Jun et al., 2004; Rajapakse et al., 2005). Usually, antioxidant peptides contain 3-16 amino acids (Li et al., 2011) with molecular masses less than 6 kDa (Sun et al., 2004). The antioxidant properties of these peptides are related to their composition, structure, and hydrophobicity (Chen et al., 1998). Peptides with aromatic residues have higher radical-scavenging ability because they can donate protons to electron deficient radicals (Sarmadi & Ismail, 2010).

Immunomodulatory activity is also a common function of bioactive peptides. Immunomodulatory peptides may be released from precursor proteins by gastrointestinal digestion and then work on the downstream immunological responses and cellular functions or by enzymatic hydrolysis *in vitro* and provide immune effects as nutraceuticals (Shahidi & Zhong, 2008). Traditionally, the major sources of immunomodulatory peptides are milk, eggs and soybean protein (Udenigwe & Aluko, 2011). Recently, immunomodulatory peptides also have been found in several new sources such as chicken and salmon (Feng et al., 2010; Yang et al., 2009).

Antimicrobial peptides may inhibit or destroy bacteria, fungi or virus particles. Some of the antimicrobial peptides have even been applied in agriculture, food manufacture and medicine (Meng et al., 2010; Lazarev & Govorun, 2010). They can be used as substitutes for common antibiotics or commercially-approved preservatives (Papagianni, 2003). Antimicrobial peptides widely exist as a first round of microbial defense in all living organisms ranging from microorganisms to plants and animals (Reddy et al., 2004). Like antihypertensive peptides, many antimicrobial peptides also share some common features. They are usually small with molecular weights less than 10 kDa. Most are cationic at neutral pH and amphipathic so that they can bind with the anionic cell surfaces of both Gram positive and Gram negative bacteria, and then kill the target cells through various

mechanisms like disrupting membranes and interfering with metabolism (Izadpanah & Gallo, 2005).

2.2.2.2 Novel functions of bioactive peptides

Bioactive peptides exhibiting anticancer activity have been discovered within the past two decades. Numerous peptides from various sources have been reported to have anticancer effects *in vivo* through different mechanisms. Table 2.3 lists some of the anticancer peptides found recent years and their functions.

Table 2.3 Peptides with anticancer activities (Shahidi & Zhong, 2008)

Peptides	Anticancer activity	Reference
Lunasin in soybean	Inhibition of carcinogens in mammalian cells	Galvez et al., 2001
Soy protein hydrolysates	Suppression of colon and liver tumorigenesis	Azuma et al., 2000 Kanamoto et al., 2001
Soy glycopeptides	Cytotoxicity to lymphoma cells	Kim et al., 1999
Buckwheat peptide	Antiproliferation to hepatoma, leukemia, and breast cancer cells	Leung et al., 2007
Ginseng peptides	Antitumor activity to lymphoma cells	Kim et al., 2003
Egg protein hydrolysates	Antiproliferation to lymphoma cells	Yi et al., 2003
Fish protein hydrolysates	Inhibition of breast cancer cells	Picot et al., 2006 Hsu et al., 2011
Algae protein	Antiproliferation to AGS cells (derive from human gastric carcinoma)	Sheih et al., 2010

Anti-obesity activity was found in soy, casein and whey protein hydrolysates (Aoyama et al., 2000a; Aoyama et al., 2000b). Pupovac and Anderson (2002) pointed out that peptides derived from soy and casein could produce the feeling of satiety by independent functions

of both opioid and cholecystokinin-A receptors in order to decrease food intake. Since obesity is closely related to several serious health problems and metabolic complications like cardiovascular disease, insulin resistance and abnormal body fat distribution, studies on anti-obesity peptides may provide new treatments for several other related diseases.

The antidiabetic function of bioactive peptides has been discovered rather recently and to date, only cod, a short-fruited bitter melon: *Momordica charantia L. Var. abbreviate*, soybean, shark and salmon were reported as potential sources (Lavigne et al., 2001; Tremblay et al., 2003; Yuan et al., 2008; Lu et al., 2011; Huang & Wu, 2010; Pilon et al., 2010). Lavigne et al. (2000, 2001) reported that cod proteins could improve glucose tolerance and insulin sensitivity in high fat-fed rats, and their studies showed that this might be attributed to certain amino acids. Yuan et al. (2008) found that the water-soluble peptide MC2-1-5 from bitter melon could reduce blood glucose level in alloxan-induced diabetic mice. Jang et al. (2010) discovered that peptides from black soybean significantly reduced blood glucose and improved glucose tolerance in db/db mice (a model of obesity, diabetes, and dyslipidemia). Huang and Wu (2010) purified and characterized an antidiabetic peptide from shark liver which reduced fasting plasma glucose level in diabetic mice. Recently, Nakaoka and his colleagues (2010) observed that the amino acid sequence "Leu-Ser-Glu-Leu" (LSEL) which was obtained by enzymatic hydrolysis of globin protein in bovine or swine haemoglobin could decrease blood glucose level and enhance insulin secretion, and the LSEL sequence exists in a variety of animals and plants (Kagawa et al., 2011). This discovery may help scientists find new sources of antidiabetic peptides using a bioinformatics approach.

2.2.3 Bioactive Peptides from Marine Fish Waste

Because of the structural diversity, marine fish are an important source of bioactive peptides. Numerous bioactive peptides have been reported from marine fish (Kim et al.,

2012; Ravichandran et al., 2010). Some marine fish also contain non-protein derived bioactive peptides such as protamine, a cationic peptide with broad range antimicrobial activity derived from herring and salmon milt (Potter et al., 2005).

Every year, the traditional marine fish processing and aquaculture industries generate vast quantities of by-products which include muscle, viscera, heads, skins, fins, frames and trimmings. These low value by-products are either discarded or used as animal feed and fertilizers that could cause environmental problems (Guerard et al., 2010). Scientists have recently paid more attention to more effective utilisation of marine by-products by value added processing technologies.

In general, the crude protein content of most fresh fish ranges from 15% to 26% (w/w) (Aberoumand, 2011). Kristinsson (2008) pointed out that 10-20% (w/w) of the total fish proteins are lost as processing waste. Therefore, marine-derived bioactive peptides may be recovered from a wide variety of high protein marine by-products, including frame material and trimmings or “cutoffs” that are produced as a consequence of the filleting operation. Potentially valuable fish-waste derived protein hydrolysates / peptides and their functions are summarized in Table 2.4.

Table 2.4 Marine fish waste derived protein hydrolysates / peptides (Harnedy & FitzGerald, 2011)

Name	Origin	Biological activity	Reference
Cod	Frame	Antioxidant ACE inhibitory	Jeon et al., 1999
Herring (*clupea harengus*)	Whole, body, head, gonads	Antioxidant	Santhivel et al., 2003
Hoki (*Johnius belengerii*)	Skin	Antioxidant	Mendis et al., 2005
	Bone	Ca-binding	Jung et al., 2005(1)
	Frame	Antioxidant	Kim et al., 2007
		Ca-binding	Jung & Kim, 2007
Pollack (*Theragra chalcogramma*)	Skin	Antioxidant	Kim et al., 2001
		ACE inhibitory	Byun & Kim, 2001
	Frame	ACE inhibitory	Je et al., 2004
		Antioxidant	Je et al., 2005
		Ca-binding	Jung et al., 2006
Sea Bream	Scale	ACE inhibitory	Fahmi et al., 2004
Snapper (*Priacanthus macracanthus*)	Skin	Antioxidant	Phanturat et al., 2010
Snapper (*Lutjanus vitta*)	Skin	Antioxidant	Khantaphant & Benjakul, 2008
Sole (*Limanda aspera*)	Skin	Antioxidant	Gimenez et al., 2009
	Frame	Antioxidant	Jun et al., 2004
		Antihypertensive	Jung et al., 2005(2)
		Anticoagulant	Rajapakse et al., 2005
Tuna	Frame	Antioxidant	Je et al., 2007
		Antihypertensive	Lee et al., 2010
Yellowtail	Bone	Antioxidant	Morimura et al., 2002
		ACE inhibitory	Ohba et al., 2003
	Scale	Antioxidant ACE inhibitory	Ohba et al., 2003

2.3 CURRENT STATUS OF UTILIZATION OF FISH WASTE

In the traditional fish processing industry, only a small portion of fish is used for human consumption. A significant part (30%-80%, depending on species) is used to produce low value products like silage or even discarded directly (Lim & Sessa, 1995). Every year, around 30 million tons of fish waste is dumped around the world creating environmental problems and at the same time, such practices do not take advantage of potential new economic opportunities (Kristinsson & Rasco, 2000). Thus, waste utilization should be an important issue for the fish processing industry from both the regulatory standpoint and because of the potential economic advantages.

The three most common ways to use fish waste include production of fish meal/oil, manufacture of silage, and production of organic fertilizer (Gill, 2000). Traditionally, manufacturers produce fish meal and oil by comminuting (breaking fish into several small fragments) and cooking fish waste to separate oil from other materials. Fish meal is often used for feeding livestock and farmed fish while fish oils are widely used for manufacturing edible oils and fats such as fish oil supplements and margarine (Windsor, 2001), and can also be an ingredient in paints (Arvanitoyannis & Kassaveti, 2008). Fish silage which is currently also produced in some countries for animal feeding is generally manufactured by acidifying fish waste with organic acids (Gill, 2000) and partially solubilised by enzymatic hydrolysis. Fish-based fertilizer is also produced by the fermentation of fish waste. Although these conversions enhance the added-value of fish waste, their relative value is still low. Several other options have been proposed and exploited. The recovery of high-value chemical components from fish waste is a promising research area and development for the utilization of fish waste. Scientists have shown that numerous useful components can be extracted from fish waste such as enzymes, gelatine, bioactive peptides, omega-3 rich oils and DNA, all of which may be used in the pharmaceutical industry (Gill, 2000). For example, trypsin extracted from cod viscera has been used commercially in food production and cosmetic manufacture (Chun et al., 2011).

In addition, fish wastes are also potential sources of natural pigments (Arvanitoyannis & Kassaveti, 2008).

2.4 METHODOLOGY

2.4.1 Production of Protein Hydrolysates

The term "hydrolysate" is defined as a "protein that is broken down into peptides of various sizes" (Herpandi et al., 2011). Currently, the predominant method for producing protein hydrolysates is through the enzymatic hydrolysis process, especially on a laboratory scale. Chemical methods including acid and alkaline hydrolysis are also in commercial use because of their competitive price (Pasupuleti & Braun, 2010). Figure 2.2 shows a typical flow diagram for the production of protein hydrolysates. Details about these methods are discussed below.

2.4.1.1 Chemical methods

Chemical digestion of protein refers to acid or alkaline-hydrolysis. Acid hydrolysis is often used for analysis of amino acid composition of protein since it does not cause racemization and destruction of serine, threonine, arginine and cysteine. However, tryptophan is destroyed by acid hydrolysis and needs to be estimated by other methods (Garrett & Grisham, 2005). The principle of acid hydrolysis is shown below (Figure 2.3). Acid hydrolysis of fish proteins can be achieved at 118°C for 18 h with 6 M hydrochloric acid (HCl) (Thomas & Loffler, 1994). Generally, acid hydrolysis is used to break down proteins into peptides and free amino acids that are widely used as flavour enhancers in the food and pet food industries (Nagodawithana, 1998; Nagodawithana et al., 2010). Compared with acid hydrolysis, alkaline hydrolysis is not used so often because it can destroy a variety of amino acids such as serine and threonine (Fountoulakis, 1998). Since both acid and alkaline hydrolysis destroys specific amino acids (Webster et al., 1982) and introduce significant amounts of inorganic salt, the utilization of chemical methods is limited.

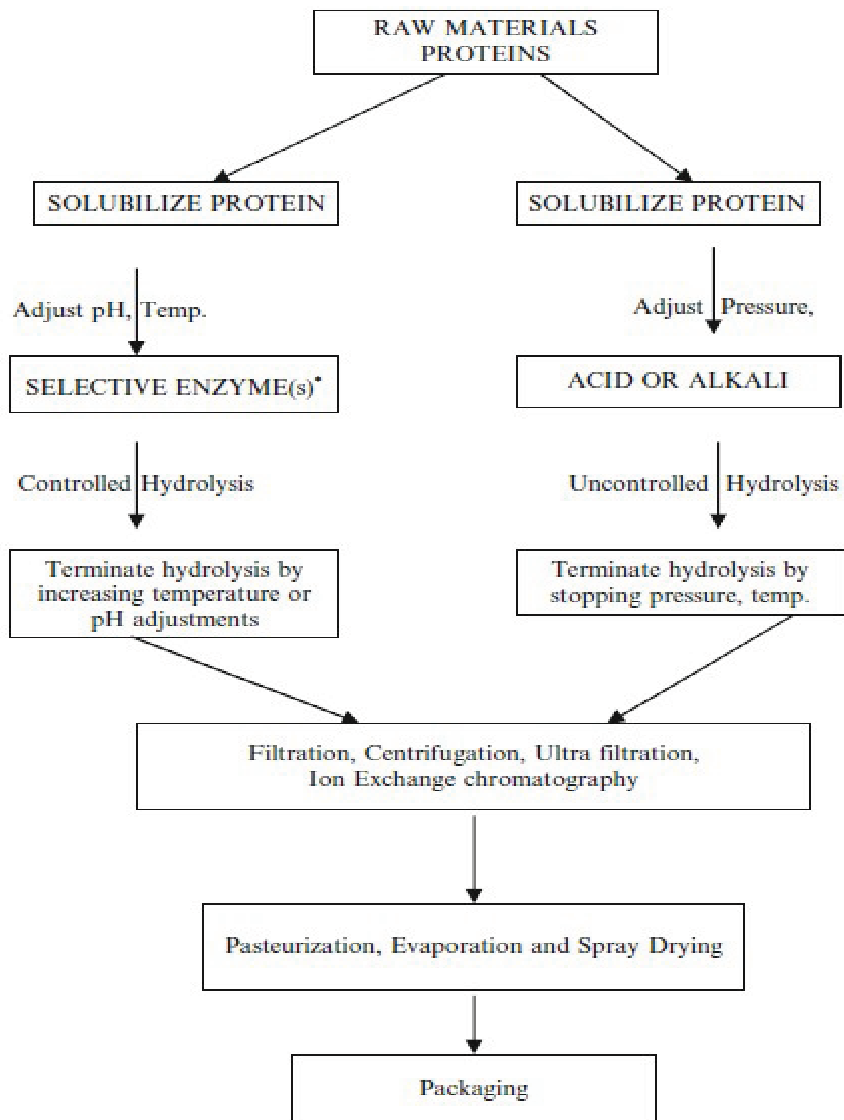

Figure 2.2 A flow chart of the typical production of protein hydrolysates (Pasupuleti & Braun, 2010, with permission)

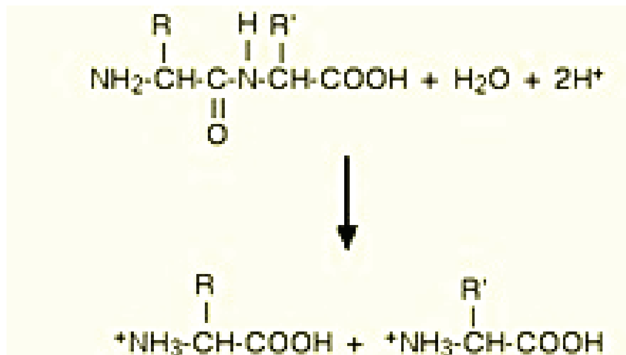

Figure 2.3 The principle of acid hydrolysis of protein (Petsko & Ringe, 2004, with permission)

2.4.1.2 Enzymatic hydrolysis

Enzymatic hydrolysis has numerous advantages:

- Enzyme hydrolysis can preserve the bioactivity of the constituent peptides without the destruction of amino acids (Wu & Ding, 2002).
- Enzymatic hydrolysis may be specific for only certain types of amino acids adjacent to the peptide bonds (Pasupuleti & Demain, 2010). Thus, the degree of hydrolysis can be controlled.
- Enzymatic digestion does not involve organic solvents or toxic chemicals so that it is suitable for the food and pharmaceutical industries (Kim & Wijesekara, 2010).
- Enzymatic digestion can be made to simulate human digestion. Thus enzymatic digestion is popular and used in the present study.

However, most of the commercial enzymes are relatively expensive which limits the use of enzymatic hydrolysis on a commercial scale.

Enzymatic hydrolysis of fish protein can be achieved by using endogenous or exogenous enzymes (Guerard et al., 2001). Exogenous enzymatic hydrolysis is more commonly used since it is easier to control and can simulate human digestion. The most commonly used

proteolytic enzymes are pancreatin, trypsin, pepsin, papain, bromelain, and bacterial and fungal proteases (Pasupuleti & Braun, 2010). The selection of enzyme depends on the substrate and final product requirements. The progress of enzymatic hydrolysis can be affected by several factors, such as enzyme selectivity, temperature, pH, reaction time and enzyme:substrate ratio. Among these, enzyme selectivity is the most important factor. Different enzymes have different specificities which will result in the production of different peptides. Table 2.5 lists the cleavage specificities of several important proteolytic enzymes. Table 2.6 lists the optimal conditions for the widely used proteolytic enzymes. In addition, since most enzymes are relatively expensive, the enzyme:substrate ratios and reaction conditions require optimisation.

Besides these traditional methods, there is a trend to use immobilized enzymes or membrane bioreactor systems to reduce the cost of the enzyme and increase the yield of products (Holownia, 2008). In addition, fermentation technology is also a way to produce diverse dipeptides and oligopeptides (Yagasaki, 2009).

Table 2.5 The cleavage specificities of common proteolytic enzymes (Liu & Zhao, 2010; Hui et al., 1996)

Enzyme	Specificity
Pepsin	Phe, Tyr, Trp, Leu
Chymotrypsin	Trp, Tyr, Phe
Trysin	Arg, Lys
Alcalase	Glu, Met, Leu, Tyr, Lys, Gln
Papain	Broad specificity, but preference for an amino acid bearing a large hydrophobic side chain at the P2 position

Table 2.6 Optimum reaction conditions of common proteolytic enzymes (Qian et al., 2007; Kechaou et al., 2008)

Enzyme	pH	Temperature (°C)
Alcalase™	7.0-8.5	50
Chymotrypsin	8.0	37
Papain	6.0	37
Pepsin	2.0	37
Neutrase™	8.0	50
Trypsin	8.0	37
Flavourzyme™	8.0	50

2.4.2 Separation and Purification of Bioactive Peptides

Based on the physical and chemical characteristics like molecular size, charge, polarity, solubility and specific covalent or non-covalent interactions, peptides can be separated and purified. The common methods for separating and purifying peptide fractions are listed in Table 2.7. The selection of separation and purification strategies is based on the characteristics of the target peptides.

Table 2.7 Common methods for separation of peptide mixtures (Allen, 1981; Sewald & Jakubke, 2009)

Technique	Examples of materials used	Properties of peptide molecules exploited
Precipitation	Ammonium sulfate, alcohol, isoelectric precipitation	Solubility, hydrophobicity
Gel filtration	Sephadex, Superdex, Bio-Gel P-2	Size, shape
Ultrafiltration	Ultrafiltration membrane	Size, shape
Dialysis	Semi-permeable membranes	Size, shape
Electrodialysis	Ion Selective membranes	Size, shape and charge
Ion exchange chromatography	Derivatives of cellulose or Sephadex (DEAE, CM, QAE, SP, phosphor-)	Charge, with some influence of polarity
Affinity chromatography	Antibody-Sepharose	Specific inter-actions with antigens
Hydrophobic interaction chromatography	Phenyl Sepharose, Octyl Sepharose	Hydrophobicity
Reverse-phase high performance liquid chromatography	C-18, C-12	Hydrophobicity

Precipitation is a preliminary separation method. It is difficult to get pure peptides by precipitation. Precipitation separates soluble peptides from insoluble ones by adding ammonium sulfate, alcohol or adjusting pH. Generally speaking, relatively large, non-polar peptides tend to be insoluble in water, especially at their iso-ionic points (Allen, 1981). Precipitation may also be useful to remove intact proteins from the peptides formed during hydrolysis. Adjusting pH to the iso-ionic point of the target peptide is a useful separation method. Kawauchi et al. (1986) utilized this technique to purify a crude growth hormone from chum salmon (*Oncorhynchus keta*). Taskaya et al. (2009) recovered muscle proteins from sliver carp based on isoelectric precipitation. Although precipitation is just a preliminary approach, it is very useful as an initial step for subsequent separation and purification.

Gel filtration which is based on molecular size and shape of digested products is a widely used efficient tool for peptide separation and purification. Large protein or peptide molecules are unable to diffuse into the pores and pass through the void volume of the column and are eluted first while the small peptides will enter the pores of the medium and are eluted later (Figure 2.4). Gel filtration can separate peptides with significant differences in molecular weight. Sometimes it is used as the first step to separate fish peptide fractions on the laboratorial-scale (Hsu, 2010; Bougatel et al., 2010; Slizyte et al., 2005). In this approach, peptide mixtures are separated into different fractions or categories based upon size. Fractions are tested for bioactivity. Then active fractions are segregated from non-active fractions, and subsequently separated by other means. However, it is impossible to separate a target peptide from a mixture of peptides with similar sizes and shapes by gel filtration.

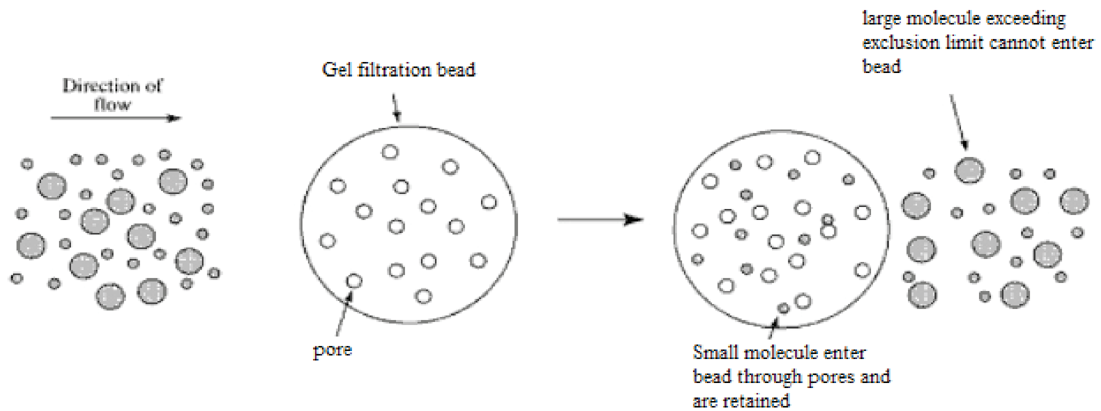

Figure 2.4 The principle of gel filtration (Sheehan, 2009, with permission).

Ultrafiltration also separates peptides based upon sizes and shape. Ultrafiltration membranes work as selective barriers. They can retain molecules that are too large to pass through the pores of the membrane while letting small molecules pass through (Baker et al., 1991). Thus, by choosing membranes with the proper pore size, target peptides can be separated. Unlike other isolation methods which are only effective on a laboratory scale, ultrafiltration has been found to be the most suitable technology for commercial scale-up (Korhonen & Pihlanto, 2006). Researchers also have studied combining ultrafiltration with other technologies to extend its application. For instance, ultrafiltration combined with enzyme reactors to produce specific peptides continuously or with electrical fields to separate peptide fractions based on the sizes and charges of samples (Bazinet & Firdaous, 2009). Unfortunately, ultrafiltration membranes are not efficacious for separation of peptides with molecular weight less than 1 kDa.

Dialysis is a process of separating molecules by their different diffusion rate through a semi-permeable membrane (Reed et al., 2007). It is typically used in separating small molecules like salts from large biological molecules such as protein and polysaccharides. Usually, a dialysis bag is filled with a soluble mixture of proteins, peptides and salts, and

when the dialysis bag is placed in a large volume solution of different composition to the contents of the bag, the smaller components from the bag including ions and small peptides and proteins, would be exchanged with ions in the dialysis tank, while the larger molecules are retained in the dialysis bag and unable to diffuse because of the uniform restrictive pore size of the semi-permeable membrane (Berg et al., 2007). However, this technique is slow, relatively unselective and generally unapplicable to commercial scale production (Hames, 1998)

The separation of peptides by gel filtration, ultrafiltration and dialysis are all size-based, which make them ineffective for small peptides or peptides with similar molecular sizes. In this case, other separation methods are required.

Ion exchange chromatography can separate peptides which have similar molecular weight but with different surface charges. It is one of the most frequently used chromatographic techniques in separation and purification of peptides because of its "wide applicability, high resolving powder and capacity, simplicity and controllability" (Amersham Biosciences, 2002). Ion-exchange separation depends on reversible competitive binding (Sivasankar, 2005). The principle of this method is shown in Figure 2.5. By controlling the pH and ionic strength of the eluting buffer, peptide fractions with different charges can be separated. Ion exchange columns have numerous applications in separating and purifying fish peptide fractions. Kim et al. (2007) used this method to purify antioxidant peptides from hoki. Gildberg et al. (1996) preferred cation exchange chromatography to isolate acid peptides fractions from Atlantic cod. Unlike gel filtration, ion exchange chromatography tends to concentrate dilute samples (Cohen & Schure, 2008). However, ion exchange separation will introduce salts because of the use of salt gradients for sample elution. The salt gradient subsequently contaminates all eluted fractions and must be removed before subsequent purification can take place. This does not present a problem when dealing with proteins

because the proteins could be easily de-salted using gel filtration or ultrafiltration strategies. However, low molecular weight (LMW) peptides (<1 kDa) are difficult to de-salt because of the similarity of size of the peptides and their contaminating salts.

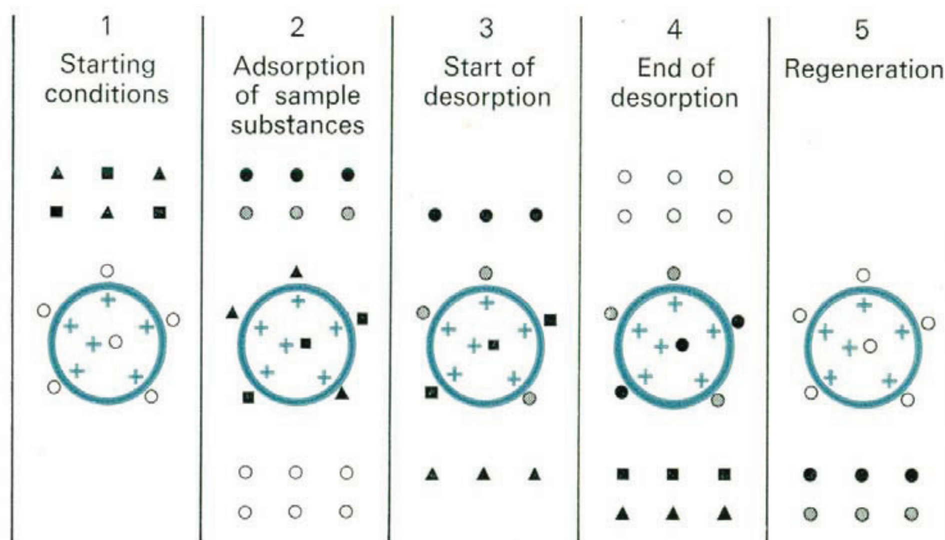

Figure 2.5 The principle of ion exchange chromatography (salt gradient elution) (Amersham Biosciences, 2002, with permission).

Affinity chromatography is quite useful if a high degree of specificity is required. In this technique, a biospecific ligand is coupled to a chromatographic bed material like agarose gels, glass beads, polyacrylamide or cross-linked dextrans. As long as the affinity for peptide and immobilized ligand is high, the peptide can be retained on the column and all the other contaminants eluted with an initial wash of starting buffer. Subsequent elution of the peptide may be accomplished using buffers containing ions which compete with the peptide-ligand complex. Compared with other techniques, affinity chromatography has numerous advantages. Most important is the fact that it often requires only one step to give a relatively pure sample and provides rapid purifications ranging from several to several

thousand-fold, and this makes it suitable for large-scale peptide purification if a relevant ligand and high capacity affinity column exist (Zachariou, 2008). In 1975, Idler and his colleagues used affinity chromatography to isolate and purify gonadotropin(s) from Chum salmon (Idler, 1975).

Hydrophobic interaction chromatography (HIC) is not as specific as affinity chromatography but it is still a powerful technique for separation and purification of proteins. The hydrophobic peptides will bind with hydrophobic ligands which are fixed on inert chromatographic supports while other less hydrophobic molecules pass through with shorter retention times (Kastner, 2000). Mechanistically opposite to ion exchange chromatography, HIC is performed using a high salt concentration at the beginning of a run to promote hydrophobic binding of proteins to the column packing material. The proteins slowly eluted as the salt concentration in the eluent buffer is lowered. Thus desalting of eluting fractions is also necessary for HIC. The recoveries of HIC are often relatively low (Michael, 2000).

Reverse-phase high-performance liquid chromatography (RP-HPLC) is similar to HIC, but is much more efficient (Amersham Pharmacia Biotech, 2000). The development of RP-HPLC is perhaps the most significant advance to date in the methodology of purifying peptides (Conlon, 2007). Now, RP-HPLC has become a necessary tool for separating and purifying peptides. It has high resolution and recovery and can be used to separate peptides with different structures as well as very closely related molecules (Aguilar, 2004). It also has good reproducibility because of the stability of the sorbent materials (Aguilar, 1996). C_8 and C_{18} columns are most often used for peptide separations with gradient elution using organic solvents such as acetonitrile or methanol and containing an ion-pairing reagent like trifluoroacetic acid (TFA) (Snyder et al., 1997). Detection is accomplished using UV absorbance at 214-220 nm in which all the components containing a -CONH- peptide bond

can be detected (Conlon, 2007). RP-HPLC is generally used to evaluate the efficacy of all of the aforementioned procedures as well as to evaluate purities of the fractions recovered. Capillary RP-HPLC is also used in tandem with mass spectroscopy to identify and sequence bioactive peptides.

2.4.3 Identification of Bioactive Peptides

Mass spectrometry (MS) followed by computational methods is currently the predominant technology for identifying peptides. A general peptide identification scheme is:

- Purification of peptide samples using suitable separation methods mentioned above.
- Peptide characterization by MS analysis (Tandem MS, Electrospray Ionization Tandem MS or Matrix-assisted laser desorption/ionisation-time of flight MS). Generally, MS is combined with a high-resolution separation technique like capillary HPLC for identification.
- Protein database screening for possible peptide sequences.
- Synthesis of target peptide.
- Confirmation of bioactivity of the identified peptide by cellular or animal assays (Mozzi et al., 2010). Besides the MS approach, the bioassay is also an effective way for identifying peptides, and for some specific peptides, receptor-based assays also work well. Peptides can be identified by their ability to bind to a known receptor and then to displace a ligand or to produce a biological response (Brady, 2011).

CHAPTER 3. OBJECTIVES

The main objectives of our project are to identify peptides from cod and salmon protein hydrolysates with antidiabetic activity and find suitable process conditions for the subsequent large-scale production of target peptides. To achieve these goals, this thesis focused on:

1) Development of a strategy to recover bioactive peptides from cod and salmon mince
2) Scale-up the process to recover bioactive peptides (MW<1 kDa) from cod and salmon mince and study the process conditions for reducing the production cost
3) Further separation and purification of the bioactive peptides (MW<1 kDa) to find the most effective peptides or peptide groups
4) Identification of the purified peptides

CHAPTER 4. MATERIALS AND METHODS

4.1 SAMPLE PREPARATION

Frozen salmon (*Salmo salar*) and cod (*Gadus morhua*) frames were received in insulated containers from Cooke Aquaculture (Saint John, NB). Both shipments were thawed at 4°C overnight and mechanically deboned using a Bibun SDX meat-bone separator equipped with a drum with 5 mm mesh holes (Bibun, Fukuyama, Hiroshima, Japan). The minced fish within the drum was collected and portions were individually frozen in polyethylene bags at -30°C until use.

4.2 LAB SCALE ENZYMATIC DIGESTION OF SALMON AND COD MUSCLE PROTEINS

At the beginning of the study, different protein extraction methods and enzymes were tested in order to fully solubilise fish proteins and to subsequently optimize the production of fish protein hydrolysates (FPH) with bioactivity. Thawed minced salmon or cod muscle tissues were mixed with 0.1 M sodium hydroxide (NaOH), 1.0 M NaOH or distilled water (dH_2O). Pepsin, pancreatin, trypsin and chymotrypsin were used alone or in combination to hydrolyze fish protein. Three different protocols were selected for FPH production (Figure 4.1). Details were given below.

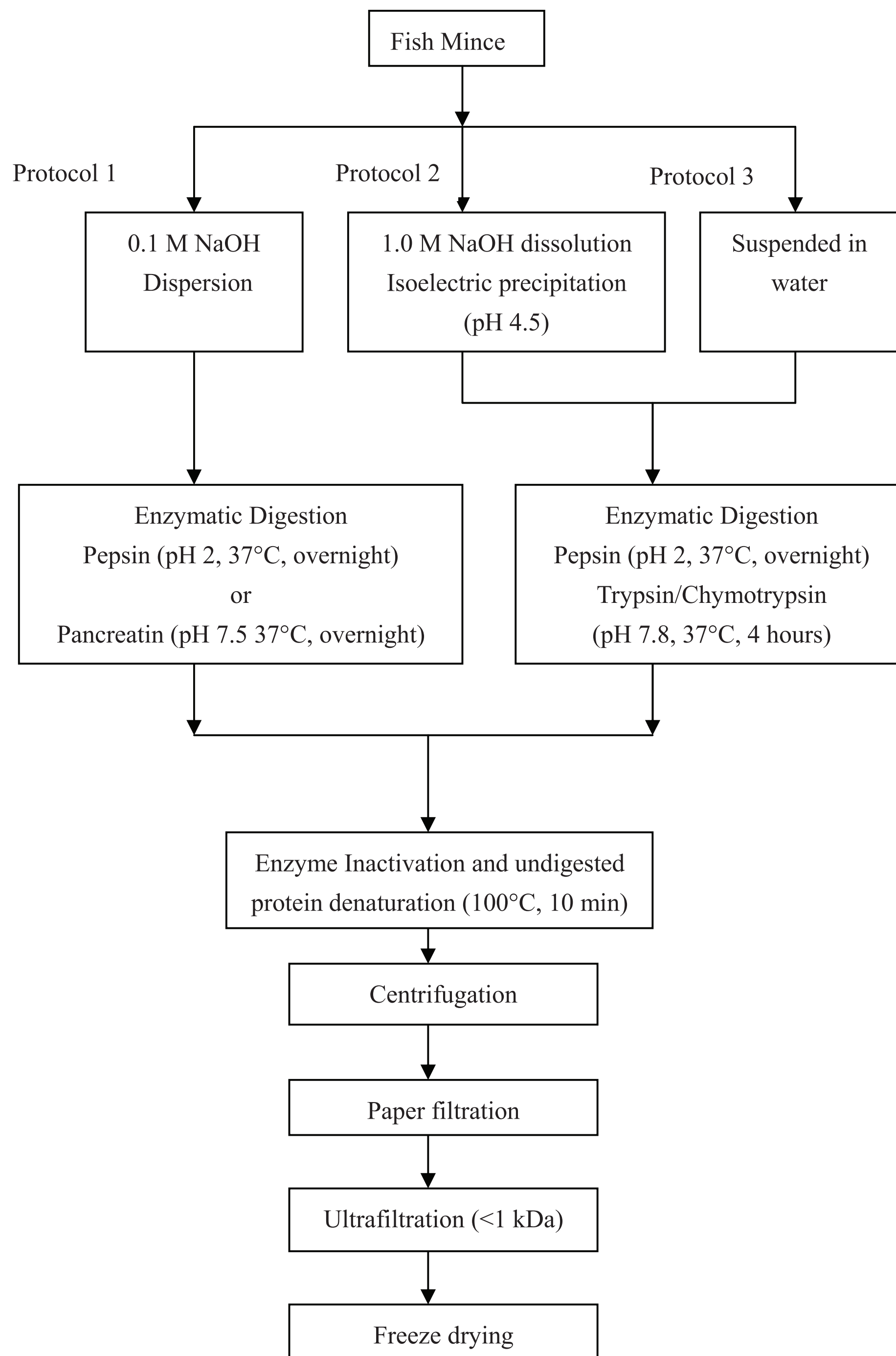

Figure 4.1 Protocols for producing salmon and cod protein hydrolysates

4.2.1 Protocol 1 (0.1 M NaOH dissolution; pepsin / pancreatin digestion)

Fifty g minced salmon or cod muscle tissue were defrosted at 4°C and then suspended in 200 mL 0.1 M NaOH. The mixture was homogenized in a standard Waring Blender for about 2 min at high speed and then stirred overnight at 4°C. The pH was adjusted to 2.0 with 2.0 M HCl for pepsin digestion or to 7.5 with 2.0 M NaOH for pancreatin digestion. Pepsin (EC 232-629-3, Sigma Cat. No.: P7125, 600-1800 units/mg protein) or pancreatin (EC 232-468-9, Sigma Cat. No.: P8096. Batch: 068k0712) was added at the enzyme:substrate (E:S) ratio: 1:100, assuming the protein contents in salmon and cod mince were 15% w/w and 20% w/w, respectively and the average activity of this pepsin was 1000 units/mg protein in all the following experiments. Thus, for 50 g salmon mince, the required pepsin/pancreatin amount was calculated as:

Pepsin amount = 50 g × 0.15 /100 =0.075 g =75 mg

and the related enzyme concentration was:

Enzyme concentration= 75 mg × 1000 units/mg / (50 g × 0.15) =10,000 units/g fish protein.

For 50 g cod mince, the required pepsin/pancreatin amount was calculated in the same way to be 100 mg. The required amounts of porcine pepsin or pancreatin were dissolved in 10 mL dH_2O, then added to those pH-adjusted fish protein dispersions. Enzyme-substrate mixtures were continuously stirred overnight at 37°C. Reactions were terminated by heating to 100°C for 10 min. The digests were centrifuged at 7000 × g for 20 min at 4°C in a fixed angle rotor and the floating lipid removed by aspiration. The supernate was then filtered using a Whatman #1 filter paper (Whatman International Ltd. Maidstone, England) to remove any insoluble material. The clarified pepsin/pancreatin digests were run through an Amicon stirred cell ultrafiltration unit (Millipore, Corporation, Bedford, MA, USA) with a 1 kDa ultrafiltration membrane. Permeates containing peptides with MW less than 1 kDa were collected and freeze-dried. The lyophilized samples were ground into powder using a mortar and pestle and then stored at -20°C until further analysis.

4.2.2 Protocol 2 (1.0 M NaOH dissolution; pepsin, trypsin and chymotrypsin digestion)

Fifty g mechanically-deboned salmon or cod mince were mixed with 200 mL 1.0 M NaOH and homogenized in a standard Waring Blender for about 2 min, then stirred for 1 h. The pH was then adjusted to 4.5 using 2.0 M HCl in order to isoelectrically precipitate the fish protein. The isoelectric precipitate was kept at 4°C overnight to make sure the precipitation was complete. The precipitated proteins were pelleted by centrifuging at 7000 × g for 20 min at 4°C. The supernate was discarded and the pellet was re-suspended in 200 mL of dH_2O with vigorous shaking. The pH of this protein dispersion was adjusted to 2.0 using 2.0 M HCl in preparation for pepsin digestion as described above under Protocol 1.

The pH of this pepsin digested sample was then adjusted to 7.8 using 2.0 M NaOH to irreversibly deactivate the pepsin (Schlamowitz et al., 1963), and to prepare for digestion with trypsin and chymotrypsin. Trypsin (EC 232-650-8, Sigma Cat. No.: T7409, 1900 units/mg powder) and chymotrypsin (EC 232-671-2, Sigma Cat. No.: C4129, 57.24 units/mg powder) were also added at E:S ratio of 1:100. Thus following the same logic as Protocol 1, the amounts of trypsin and chymotrypsin used for 50 g salmon were 75 and 75 mg, respectively, while for 50 g cod, the amounts were 100 and 100 mg, respectively. After adding trypsin and chymotrypsin, the pepsin digested sample was placed in a 37°C incubator with gentle stirring for 4 h. Then, the hydrolyzed fish proteins were clarified, ultrafiltered and freeze dried as described in Protocol 1. The freeze-dried powders were also stored at -20°C until further analysis.

4.2.3 Protocol 3 (Water dispersion; pepsin, trypsin and chymotrypsin digestion)

Fifty g thawed salmon or cod mince was weighed into a 1 L beaker, suspended in 200 mL dH_2O, and homogenized in a standard Waring Blender for about 2 min at high speed, then stirred in the cold room overnight. All the subsequent procedures were exactly the same as

described in Protocol 2, above.

The bioactivities of all the salmon and cod protein hydrolysate powders from protocols 1, 2 and 3 were determined *in vitro* using cell culture-based screening at Laval University or in Dalhousie's Department of Biochemistry and Molecular Biology. The effects of these LMW (<1 kDa) FPHs were tested on glucose metabolism, insulin action and lipid/lipoprotein metabolism by using L6 myocytes, HepG2 hepatic cells, macrophages and 3T3-L1 adipocytes. (Lavigne et al., 2001; Pilon et al., 2004; Miller et al., 2008).

4.3 PROCESS SCALE UP

The bioactivity tests indicated that the salmon protein hydrolysate (<1 kDa) produced using ***Protocol 2*** and cod protein hydrolysate (<1 kDa) produced by ***Protocol 3*** had the highest bioactivities as measured using glucose uptake in mouse L6 myocytes. In order to prepare more material for animal feeding trials, a scaled-up production strategy for salmon protein hydrolysate (<1 kDa) was developed. Most of the effort to date has been focused on salmon peptides because of the relative availability of farmed salmon frames and the limited availability of aquaculture cod tissues.

4.3.1 Two hundred and fifty g batch

A two hundred and fifty g sample of salmon mince was homogenized in 1 L 1.0 M NaOH. The initial mixing under alkaline conditions was done to solubilise the maximum amount of muscle protein. The isoelectric precipitation and enzymatic hydrolysis of salmon were carried out following Protocol 2. The digested samples were centrifuged at 5200 × g for 30 min at 4°C in a Sorvall RC-3 refrigerated centrifuge (Sorvall Instruments Div., Dupont Co., Newtown, CT, USA) and the supernate filtered using Whatman #1 qualitative filter paper. The filtrate was subsequently ultrafiltered using a Prep / Scale Tangential Flow Filtration

(TFF) 2.5 ft^2 cartridge with 1 kDa exclusion limit (Millipore Corporation, Bedford, MA, USA). The ultrafiltration apparatus was set up as Figure 4.2. The permeate fraction (MW<1 kDa) was collected and freeze dried. The freeze dried samples were stored at -20°C until further use. The 250 g batch production was repeated three times.

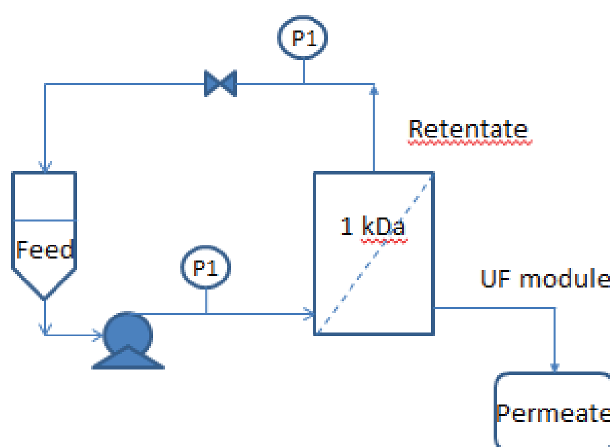

Figure 4.2 Schematic diagram of the TFF filtration apparatus

4.3.2 Two thousand five hundred g batch

A two thousand five hundred g batch process was then developed for salmon peptide production. Two thousand five hundred g salmon mince was placed in a 14 L glass digester and 1.0 M NaOH added to fish mince to a total volume of 10 L. Protocol 2 was carried out for isoelectric precipitation and enzyme hydrolysis. Digestion temperature was maintained at 37°C using a Neslab Endocal refrigerated circulating water bath (Neslab Instruments, Inc., Portsmouth, USA) and stirring accomplished with a stainless steel impeller. Enzyme inactivation was carried out by heating to 100°C for 10 min in a stainless steel steam jacketed cooker (Southbend, NC, USA). The digests were cooled and centrifuged at 5200 × g for 30 min at 4°C, then filtered using a plate and frame Buon Vino Super Jet filter press

equipped with #3, 0.5 µm pads (Buon Vino, Inc., Cambridge, ON, Canada). The filtered digests were then ultrafiltered and the permeate (< 1 kDa) freeze-dried. This 2500 g production was repeated twice.

4.3.3 Twelve and one half kg batch

A twelve and one half kg batch was produced in the food processing pilot plant at Dalhousie University. Twelve thousand, five hundred g salmon mince was placed in a Bordeau TRT-30 steam jacketed stainless steel kettle, and suspended in 1.0 M NaOH to a total volume of 50 L. The mixture was stirred to dissolve the protein using a stainless steel impeller for 2 h at high speed (Lightnin mixer Model LIU10, Wytheville, Virginia, US). Six molar HCl was used to adjust the pH of this solution to 4.5 for isoelectric precipitation and the solution was kept overnight in the 4°C cold room to ensure the precipitation was complete. The precipitated salmon protein was pelleted by centrifugation at 5200 × g for 30 minutes at 4°C using a 4-place, 4 L swinging bucket rotor and the supernate (including lipid) was removed by aspiration and discarded. The isoelectric pellet was resuspended in a minimal quantity of distilled water to a total volume not exceeding ~50 L and pH was adjusted to 2.0 with 6.0 M HCl. A pepsin: substrate ratio of 1:100 (18.75 g, EC 232-629-3; Sigma P7125, 600-1800 units/mg protein) was used for the initial pepsin digestion. The digest was routinely gently stirred overnight at 37°C. Considering the trypsin and chymotrypsin used for the small scale production were expensive, a lower cost, commercially available blend of trypsin and chymotrypsin (Enzeco Chymotrypsin® 1:1, trypsin: 1000 units/mg protein, chymotrypsin: 1000 units/mg protein, Enzyme Development Corporation, NY) was substituted. The pH of the pepsin digest was adjusted to 8.0 using 8.0 M NaOH and 18.75 g of this enzyme blend was added, giving a final E:S ratio of 1:100. Although the commercial grade trypsin concentration was lower than previously produced smaller batches, the chymotrypsin concentration was somewhat higher for the commercial grade product. The digestion was carried out at 37°C with gentle

stirring for 4 h. Then the digest was heated to 100°C for 10 min to inactivate the enzyme. The subsequent steps for the 12.5 kg batch followed the same procedure outlined above for the 2500 g batch. The filtered digests were then ultrafiltered and the permeate (<1 kDa) freeze-dried. The 12.5 kg batch process was carried out at least ten times.

4.4 TESTING REPRODUCIBILITY OF PEPTIDE PRODUCTION

The <1 kDa salmon protein hydrolysates prepared from one 50 g batch, one 250 g batch and three 12.5 kg batches were randomly selected in order to test the reproducibility of ***Protocol 2*** and the feasibility of further scale up of this process. Samples from these 5 randomly-selected batches were tested for homogeneity using reverse phase high performance liquid chromatography. These five samples were dissolved in dH_2O with 0.1% TFA (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 20 mg/mL, and then filtered with Whatman 0.2 µm syringe filters (GE Healthcare, Mississauga, ON, Canada) prior to injection. Samples were loaded onto an X-Bridge BEH 130 C_{18} peptide column (5 µm, 4.6×150 mm) (Waters, Milford, MA, USA) using a Waters analytical HPLC system (Waters, Milford, MA, USA), and eluted with a binary gradient where solvent A was water with 0.1% TFA and solvent B was 100% methanol with 0.1% TFA. The elution gradient is presented in Table 4.1. Forty µL of each sample was injected and eluted at a flow rate of 1.0 mL/min. Peptides were detected at 215 nm with a Waters 996 Photodiode Array (PDA) detector (Waters, Milford, MA, USA). The data were collected and chromatograms were compared as analyzed by the Millennium Chromatography Manager Software v 3.2.

Table 4.1 The elution gradient for HPLC of peptides

Time (min)	%A	%B
0	100	0
70	60	40
73	0	100
78	100	0
90	100	0

4.5 DETERMINATION OF NITROGEN CONTENT AND RECOVERY

The nitrogen contents were monitored at each step in the production of the FPHs from salmon and cod mince as measured by Kjeldahl analysis according to the Büchi Kjeldahl Guide (Egli, 2008). The nitrogen content of each sample was determined by titration with HCl as follows:

% N = volume HCl (L) × molarity HCl (moles/L) × 14.01 g/mole / g sample × 100%.

Where the molar mass of nitrogen is 14.01 g/mole.

Then the nitrogen recovery (NR) of each batch of FPH was calculated as:

% NR= (Total nitrogen in powder / Total nitrogen in minced fish) × 100%

4.6 EFFECT OF ENZYME CONCENTRATION ON NITROGEN RECOVERY FOR SALMON DIGESTS (PROTOCOL 2)

The 250 g batch size was used to study the effect of different enzyme concentrations on the final nitrogen recovery. Pepsin (600-1800 units/mg protein) and Enzeco Chymotrypsin® 1:1 mixture were used for these experiments. Three different E:S ratios were tested (Table 4.2). The reactions were held at optimum conditions (pH 2.0 and 37°C for pepsin; pH 8.0 and

37°C for Enzeco Chymotrypsin® 1:1). The rest of the conditions were the same as used in Protocol 2. The procedure to determine the nitrogen mass balance is shown in Figure 4.3. Nitrogen recovery was recorded instead of % protein since no valid conversion factor could be determined without the molecular identification of the bioactive peptide(s). Nitrogen recovery was determined as described in Section 4.5. Peptide profiles of all salmon protein hydrolysates (<1 kDa) were examined by analytical HPLC in order to see to what extent the enzyme concentration would affect the composition of the final products.

Table 4.2 Enzyme concentrations used to determining effect on nitrogen recovery for salmon protein hydrolysate (< 1 kDa)

Concentration / **E/S ratio**	**Pepsin** (Sigma) (units/g fish protein)	**Trypsin/Chymotrypsin** (Enzeco) (units/g fish protein)
1:100	10,000	10,000
1:200	5,000	5,000
1:500	2,000	2,000

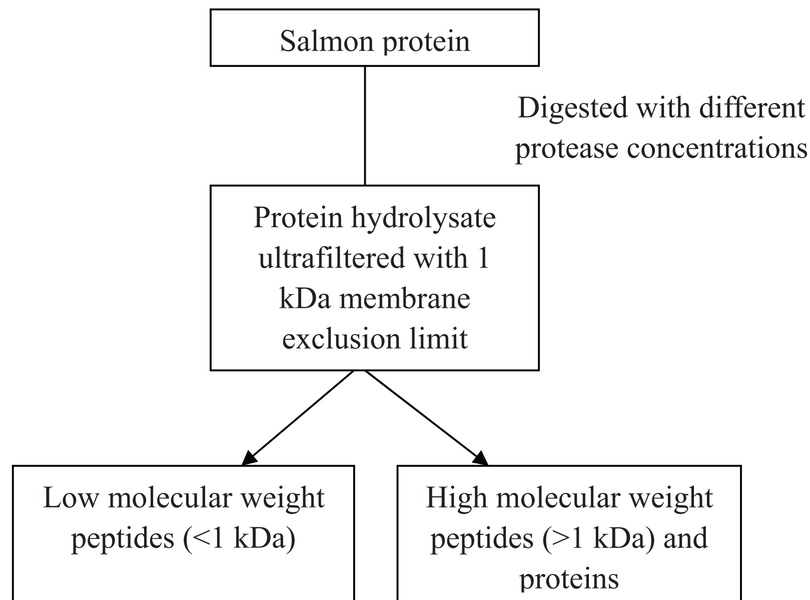

Figure 4.3 Protocol for determining the effect of different enzyme concentrations on nitrogen recovery.

4.7 OPTIMIZATION OF INCUBATION TIME FOR SALMON DIGESTION (PROTOCOL 2)

The ninhydrin assay can be used to determine the peptides and amino acids released by enzymatic hydrolysis (McGrath, 1971). The enzymatic hydrolysis of salmon protein could be considered ceased as the production of non-protein nitrogen became constant. The strategy used to monitor the generation of non-protein nitrogen was developed as a function of time as sampled at various points during the digestion.

Minced salmon digested with pepsin (600-1800 units/mg protein, Sigma) was prepared as described in Protocol 2. Five mL aliquots of digests were sampled at 0.5 h intervals for the initial 3 h and then 1 h intervals up to 9 h. An extra sample was taken at 24 h. Aliquots were immediately mixed with 5 mL 10% (w/v) trichloroacetic acid (TCA) to terminate the enzyme reaction and precipitate the undigested proteins. The mixtures were kept in the 4°C

cold room overnight for completely precipitating protein and then centrifuged at 3625 × g in an IEC Centra MP 4R centrifuge (International Equipment Company, Chattanooga, TN, USA) for 10 min. The supernate containing non-protein nitrogen was filtered with Whatman 0.45 µm syringe filters and then 0.2 mL supernate was taken out and mixed with 1 mL 0.2 M sodium acetate buffer (pH 5.5). Distilled water was added to make up to a volume of 4 mL. Then 1 mL 8% (w/v) ninhydrin (dissolved in acetone) was added. The solution was mixed and incubated for 15 min at 80°C, then cooled in ice water and mixed with 1 mL 50% ethanol in water. Absorbance was taken at 570 nm. The 0 min sample was used as a blank to zero the instrument. Since our sample is a peptide mixtures with unknown composition, there is no appropriate standard for making the standard curve, the abosobances of the samples were measured for qualitively determining the generation of non-protein nitrogen. Salmon samples were digested with three different enzyme concentrations (Table 4.2).

The pepsin digests described in Protocol 2 were used as substrates for the trypsin /chymotrypsin digestions (Enzeco Chymotrypsin® 1:1). As before, five mL aliquots of digests were sampled at 30 min intervals for 3 h and 60 min intervals for up to 7 h. The incubation time was studied following the same procedure above. Also three different enzyme concentrations were tested.

4.8 DETERMINATION OF PEPTIDE PROFILES OF THE FISH PROTEIN HYDROLYSATES

The freeze-dried salmon and cod protein hydrolysates (<1 kDa) were dissolved in dH_2O containing 0.1% TFA at a concentration of 20 mg/mL and filtered with Whatman 0.2 µm syringe filters. The peptide profiles of the fish protein hydrolysates were examined by analytical HPLC according to the conditions outlined in Section 4.4.

4.9 GEL FILTRATION OF SALMON AND COD PROTEIN HYDROLYSATES

Gel filtration chromatography was performed at room temperature using a Tricorn Superdex Peptide 10/300 GL column (Amersham Biosciences AB, Uppsala, Sweden) to estimate the molecular weight distribution of the FPHs (<1 kDa) and fractionate these peptides based upon size. The fractionation range published for this column was 100 to 7000 Da. The column was coupled to an ÄKTA Explorer 10 XT system (Amersham Biosciences AB, Uppsala, Sweden) and run with acetonitrile: H_2O: TFA = 30:70:0.1 as mobile phase. The flow rate was 0.3 mL/min, and the absorbance was monitored at 215 nm. The column was calibrated with standard peptides of known molecular weight: cytochrome C (12.5 kDa), aprotinin (6.5 kDa), bacitracin (1.4 kDa), hexa-glycine (360.3 Da), histidine (155 Da) and alanine (89 Da) (Sigma, St. Louis, MO, USA). The freeze-dried salmon and cod protein hydrolysates (<1 kDa) were dissolved in the elution buffer at a concentration of 10 mg/mL and filtered with a Whatman 0.2 μm syringe filter before injection (500 μL).

4.10 PREPARATIVE HPLC FRACTIONATION OF SALMON AND COD PROTEIN HYDROLYSATES

Preparative HPLC fractionation was carried out at the Richardson Center for Functional Foods & Nutraceuticals at the University of Manitoba. The freeze-dried salmon and cod peptides (<1 kDa) were dissolved in HPLC grade water with 0.1% TFA at a concentration of 100 mg/mL and separated on a Jupiter C_{12} column (21.2×250 mm, Phenomenex, Torrance, CA, USA) using a Varian 940-LC system (Agilent Technologies, Mississauga, ON, Canada). Four mL samples were injected each time and eluted on a binary gradient system where solvent A was water with 0.1% TFA and solvent B was 100% methanol with 0.1% TFA. The flow rate was 10 mL/min and the absorbance was monitored at 215 nm.

The elution gradients are listed in Table 4.3. Eluted fractions were pooled, collected and freeze-dried, then stored at -20°C. Freeze-dried powders were sent to Laval University for bioactivity testing.

Table 4.3 Elution gradients for preparative HPLC fractionation of fish protein hydrolysates

Fish / Time (min)	Salmon		Cod	
	%A	**%B**	**%A**	**%B**
0	100	0	100	0
35	40	60	20	80
40	0	100	0	100
50	0	100	0	100
60	100	0	100	0

4.11 ION EXCHANGE CHROMATOGRAPHY

The <1 kDa salmon and cod protein hydrolysates exhibiting the highest antidiabetic activities were also fractionated by ion-exchange chromatography. The salmon and cod protein hydrolysate powders (<1 kDa) were dissolved in 20 mM Tris-HCl buffer (pH 8.0) at a concentration of 5 mg/mL and applied to a UNOsphere™ Q anion-exchange column (10×150 mm) (Bio-Rad Laboratories, Hercules, CA, USA) (500 μL) that had been equilibrated with the same buffer. The protein hydrolysates were eluted in a linear gradient of NaCl from 0 to 1.0 M in 20 mM Tris-HCl buffer (pH 8.0) over 64 mL. The absorbance was detected at 215 nm and the flow rate was 0.5 mL/min. Eluted fractions were routinely pooled, collected and freeze-dried, then stored at -20°C waiting for bioactivity testing and further purification.

4.12 FURTHER HPLC ANALYSIS

Fractions from the Superdex Peptide 10/300 GL , Phenomenex C_{12} and UNOsphere™ Q column were again analyzed on the X-Bridge BEH 130 C_{18} peptide column (5 µm, 4.6×150 mm) using the Waters analytical HPLC system. The lyophilized fractions (20 mg/mL) were also dissolved in dH_2O containing 0.1% TFA. Samples were filtered with Whatman 0.2 µm syringe filters and eluted under the same conditions in Section 4.4.

CHAPTER 5. RESULTS AND DISCUSSION

5.1 PRODUCTION OF FISH PROTEIN HYDROLYSATE

A major objective of this study was to produce anti-diabetic peptides from salmon and cod mince on a preparative scale. The commercial-scale production process must have good reproducibility, reasonable yield of bioactive peptides and the procedure should be simple enough to perform on a large scale. The yield of each step was measured and the potential ways to increase the yield discussed.

5.1.1 Mechanical deboning of fish frames

Thawed headless salmon and cod frames were passed through the Bibun meat separator and the minced flesh was recovered for further experiments. During this process, the system loss was ~2.27%. The de-boning yields averaged 57.61±5.17% (n=5) for salmon and 45.90±4.76% (n=2) for cod, however these are likely to vary somewhat and depend on the amount of meat left on the frame material (Table 5.1). Considering the raw materials were fish frames, the relatively high deboning yields were considered excellent and suggested that perhaps higher filleting yields could have been accomplished at the plant.

Table 5.1 Deboning yields

Fish	Average starting fish frame weight (kg)	Average deboned mince weight (kg)	%Yield (Average ± SD[a])
Salmon (n[b]=5)	69.33	39.10	57.61±5.17
Cod (n[b]=2)	61.73	28.12	45.90±4.76

a: SD stands for standard deviation
b: n stands for experiment number

5.1.2 Nitrogen recovery

The peptide mixtures produced by the various protocols were tested for activity and it was not surprising to find that the highest activities were recovered from the low molecular weight material (<1 kDa) since intestinal absorption of larger proteins and peptides does not occur (Matthews, 1971). Originally, eight low molecular weight FPHs were prepared using four different hydrolysis conditions (pepsin digestion of fish proteins solubilised in 0.1 M NaOH; pancreatin digestion of fish proteins solubilised in 0.1 M NaOH; pepsin/trypsin/chymotrypsin digestion of fish proteins solubilised in water; and pepsin/trypsin/chymotrypsin digestion of fish proteins solubilised in 1.0 M NaOH) and then screened for glucose uptake in L6 mouse myocytes. From these initial experiments, the most promising peptides were contained in the FPHs obtained after solubilisation in water or 1.0 M NaOH and sequential hydrolysis with pepsin/trypsin/chymotrypsin and the low molecular weight (< 1 kDa) fractions were more active. Therefore the following studies were conducted focusing on these fractions recovered using Protocol 2 and 3.

The 250 g batch was used for studying the recovery of FPHs from fish frames. The freeze-dried salmon and cod protein hydrolysates (<1 kDa) were both white to light yellow in color without fishy odor or bitter taste. Therefore these FPHs were considered ideal for the further animal feeding experiment without need for debittering (removing the bitter components from these FPH).

For the 250 g batches, the average weight of the freeze-dried salmon and cod protein hydrolysates (<1 kDa) were 11.4 and 22.8 g, respectively. Thus the yields were approximately 4.6% and 9.1% for salmon and cod by weight, respectively. The difference between the salmon and cod yields may be due to the different protein extraction methods, the proximate composition of the two fish species and perhaps species-specific protein compositions. However, these data do not objectively reflect the recovery rate of specific

bioactive peptides from fish mince. Nitrogen content was used as a reference to determine the yield of each step and the recovery rate of FPH. The measurement of nitrogen was selected rather than protein level since the multiplication factors used to convert Kjeldahl % N into % protein are unknown for the peptide mixtures. The yields determined at each processing step and the nitrogen recoveries from fish frames are shown in Table 5.2. The nitrogen recovery from fish frames appeared to be lower, for salmon (16.9%) as compared to cod (40.1%). This does not suggest that the yield of bioactivity was higher for cod *per se,* since at this stage, the concentrations of the active ingredients in the <1 kDa peptide mixtures from cod or salmon are unknown. The *in vitro* tissue culture results also suggest that peptides produced from alkali-extracted, isoelectrically precipitated salmon tissue gave better results than did the cod. The reasons for higher nitrogen recovery observed for cod could be due to several factors such as:

- Higher fat content of salmon may decrease the efficiency of digestion.
- The salmon process was far more complicated and involved alkali solubilisation as well as isoelectric precipitation, thus more chance for nitrogen loss.
- Since salmon proteins were first fractionated by selective pH 4.5 isoelectric precipitation, larger nitrogen losses would be expected in salmon as compared to cod.

After isoelectric precipitation, 13.8% of total salmon nitrogen was lost. Generally, fish proteins have isoelectric points ranging from pH 4.5 to 5.5 (Foh, 2011). Some salmon proteins may not totally precipitate at pH 4.5 and therefore remain in the supernate which was decanted after centrifugation. In addition, the Kjeldahl method measures total nitrogen, including protein- and non-protein nitrogen, the latter including DNA, nucleotides, free amino acids, low molecular weight peptides and amines may not be precipitated by this isoelectric precipitation step. If the amino acid sequence identities of the bioactive peptides were known, one could perhaps identify their parent proteins and therefore design a process to selectively recover the bioactive peptide precursors.

After enzymatic hydrolysis, 54.2% of the total salmon nitrogen was retained. The efficiency of enzymatic hydrolysis depends on various factors such as the type and concentration of the enzyme as well as pH, temperature and incubation time (Santos et al., 2011). Optimizing these conditions is important, particularly for commercial production. Salmon protein was digested in a two-step process using pepsin and then a trypsin and chymotrypsin mixture. Although most proteins are hydrolyzed into free amino acids and peptides, it may be that one possible reason for nitrogen loss is the presence of insoluble peptide fragments containing high proportions of hydrophobic amino acid residues (Thermo Fisher Scientific, 2008), and these insoluble peptides would therefore be lost during filtration or centrifugation steps. Perhaps alternative enzymes such as Alcalase and papain could be tested or maybe this has to be accepted.

For salmon, the biggest nitrogen loss occurred during the ultrafiltration process. The yield of this step was only 33.0%. In practice, approximately 200 mL solution remained in the ultrafiltration apparatus for each run and this contained larger peptides with molecular masses higher than 1 kDa. Testing other enzymes to hydrolyze the salmon protein may increase the yields during the ultrafiltration step. However, this in turn may decrease the yield of bioactivity.

In the final freeze drying step, approximately 1% of total nitrogen was lost and was attributed to adhesion to the containers in which the peptide mixtures were placed. This loss could perhaps be reduced by placing product into containers or packaging films with hydrophobic surfaces.

In both cases, fractionation by ultrafiltration led to considerable nitrogen loss. However, this may not necessarily be a bad thing since this process in fact produced peptides with a higher level of biological activity than the unfractionated fish protein hydrolysates with

mixture of activities.

Table 5.2 Yield of each production step and the nitrogen recovery of fish protein hydrolysate.

Fish	Step	Total nitrogen (g)	Nitrogen loss (g)	Yield of each step (%)	Average nitrogen recovery (%) ±SD
Salmon (n=2)	**Salmon mince (250 g)**	6.73	0	100	100
	Salmon isoelectric precipitation	5.77	0.96	86.2	86.2±8.8
	Enzyme hydrolyzed salmon nitrogen	3.62	2.15	62.6	54.2±8.7
	Salmon protein hydrolysate ultrafiltration (<1 kDa)	1.19	2.43	33.0	17.9±3.4
	Freeze dried salmon protein hydrolysate (<1 kDa)	1.13	0.06	94.8	16.9±3.0
Cod (n=2)	**Cod mince (250 g)**	6.84	0	100	100
	Enzyme hydrolyzed cod nitrogen	6.10	0.74	89.6	89.6±10.9
	Cod protein hydrolysate ultrafiltration (<1 kDa)	3.04	3.06	49.8	44.5±2.2
	Freeze dried cod protein hydrolysate (<1 kDa)	2.74	0.30	90.3	40.1±1.6

5.1.3 Reproducibility of the scaled-up process

If the production parameters are not carefully controlled and the production of peptides lacks reproducibility, the cell culture and animal feeding trials are meaningless. Five freeze dried salmon protein hydrolysates (<1 kDa) samples were randomly selected from three different production batches (50 g, 250 g and 12.5 kg) and their peptide profiles checked by analytical RP-HPLC. The results are shown in Figure 5.1. Figure 5.1 (a) is the peptide profile of one sample from the 50 g batches, (b) is from a 250 g batch, and (c)-(e) are from three different 12.5 kg batches. These five chromatograms show that the retention times for the major peaks are similar, suggesting that at least qualitatively the peptide profiles were similar. In addition, it would appear that the Enzeco Chymotrypsin® 1:1 produced profiles (c-e) that were similar to those produced by the more expensive Sigma trypsin and chymotrypsin (a, b). Furthermore, the scaled-up (12.5 kg) protocol produced patterns that were qualitatively similar to those prepared from smaller batches of minced fish. Further quantitative experiments may be required, since it is impossible to quantitatively judge the reproducibility of the scale-up process based on these chromatograms.

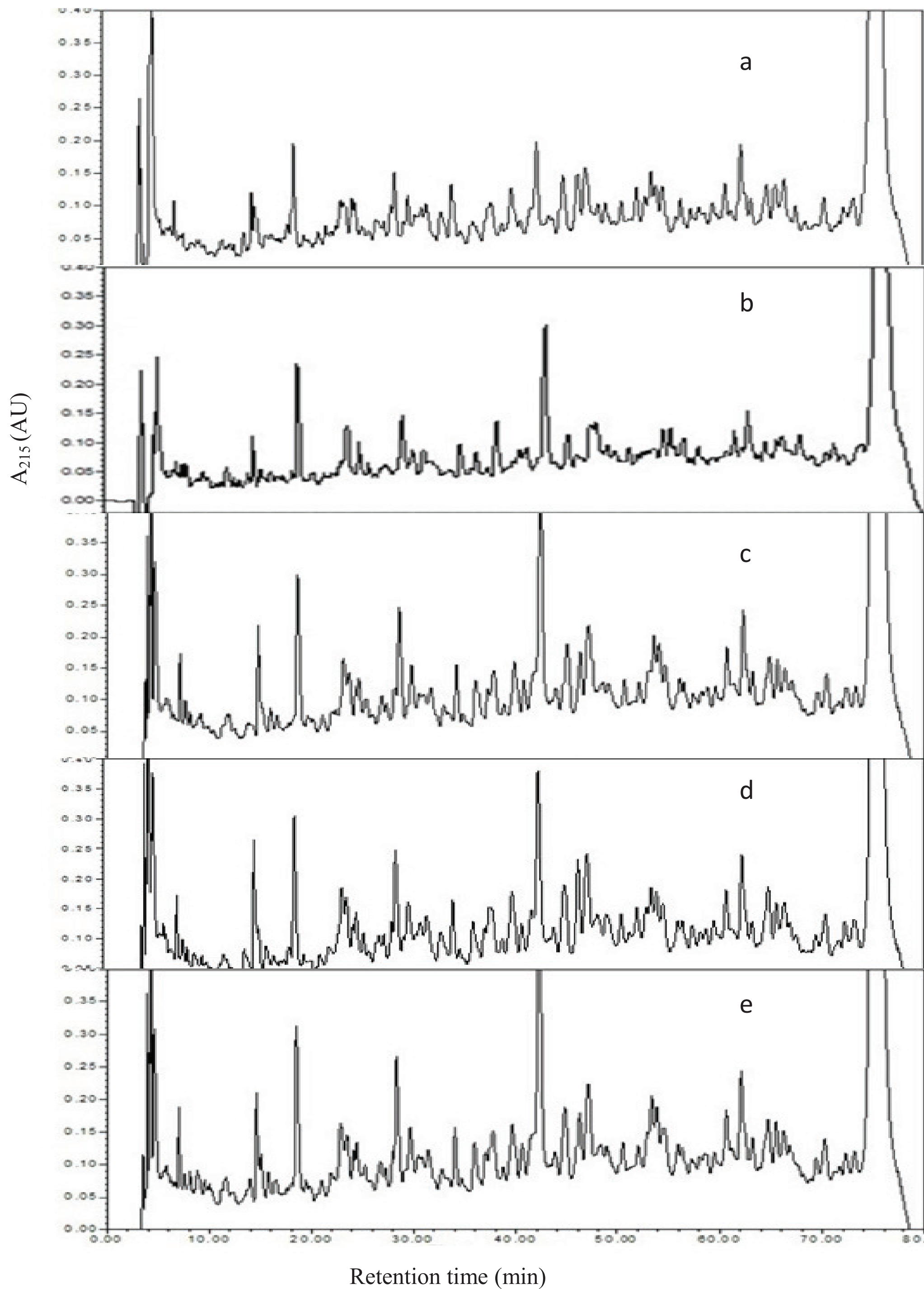

Figure 5.1 RP-HPLC peptide profiles of different batches of salmon protein hydrolysate (<1 kDa). a) 50 g batch. b) 250 g batch. c-e) three different 12.5 kg batches. Column: Waters X-Bridge BEH 130 C_{18}; eluant A, water containing 0.1% TFA; eluant B, 100% methanol containing 0.1% TFA; flow rate, 1 mL/min; detection at 215 nm.

5.1.4 The effect of enzyme concentration on nitrogen recovery

For applying the process to industrial-scale manufacture, one important thing to consider is the cost. Most enzymes are very expensive. Thus, determining the effect of enzyme concentration on the nitrogen recovery can help find an optimal enzyme concentration which could reduce the cost without a substantial reduction in nitrogen recovery. The nitrogen recoveries for enzymatically-digested minced salmon were compared for three different enzyme concentrations (Table 4.2). The results shown in Table 5.4 illustrate that for these three enzyme concentrations that were tested, decreasing the enzyme concentration, resulted in a decrease in nitrogen recovery of the final product (<1 kDa fraction). However, compared with the decrease of the enzyme concentration, the subsequent decrease in nitrogen recovery was relatively small. Table 5.4 indicates that halving of the enzyme concentration resulted in a 1.6% decrease in the final nitrogen recovery while a 5-fold reduction only reduced the nitrogen recovery by 2.7%. The statistical analysis shows that the decrease of the nitrogen recoveries among the three enzyme levels tested is linear with the decrease of the enzyme concentration, but the differences between the nitrogen recoveries are not significant ($\alpha=0.05$, $r=0.688$). Table 5.3 lists the current price of enzymes used in this study. Although compared with trypsin and chymotrypsin, pepsin is much cheaper, both of them are still expensive and will limit the application of large scale production of the bioactive peptides. Based on the preliminary results and the price of the enzymes, it seems that using lower enzyme concentrations can decrease the cost of production without a large decrease in nitrogen recovery.

In order to confirm that reduction in enzyme concentration will not influence the composition of the final products, all of the salmon protein hydrolysates (<1 kDa) were analyzed by HPLC and the chromatograms were compared. The results shown in Fig. 5.2 represent the peptide profiles of the salmon protein hydrolysates (<1 kDa) produced with (a) 10,000, (b) 5,000 and (c) 2,000 enzyme units per g salmon protein. The chromatograms

demonstrate that at least qualitatively, the three different enzyme concentrations gave similar peptide chromatographic fingerprints. However, it seems that different enzyme concentrations produced some quantitative changes for these major peaks (Fig 5.2) and these differences were demonstrated as differences in ratios of peak areas. However, to properly measure these quantitative differences, an internal standard would be required.

Table 5.3 The 2012 price of enzymes used in this study

Enzyme	Price (CAD)
Pepsin (Sigma, 600-1800 units/mg protein)	$542/kg enzyme
Trypsin/chymotrypsin (Enzeco chymotrypsin®1:1, 1000 units/mg protein)	$3000/kg enzyme

Table 5.4 Nitrogen recoveries of salmon hydrolysates using different enzyme concentrations (pepsin/trypsin/chymotrypsin)

Enzyme concentration (enzyme units/g salmon protein)	Average initial nitrogen content (g)	Final nitrogen content in freeze dried powder (MW <1 kDa) (g)	Nitrogen recovery (%)
10,000 (n=2)	6.73	1.13	16.8±1.2
5,000 (n=2)	6.73	1.02	15.2±1.3
2,000 (n=2)	6.73	0.95	14.1±2.4

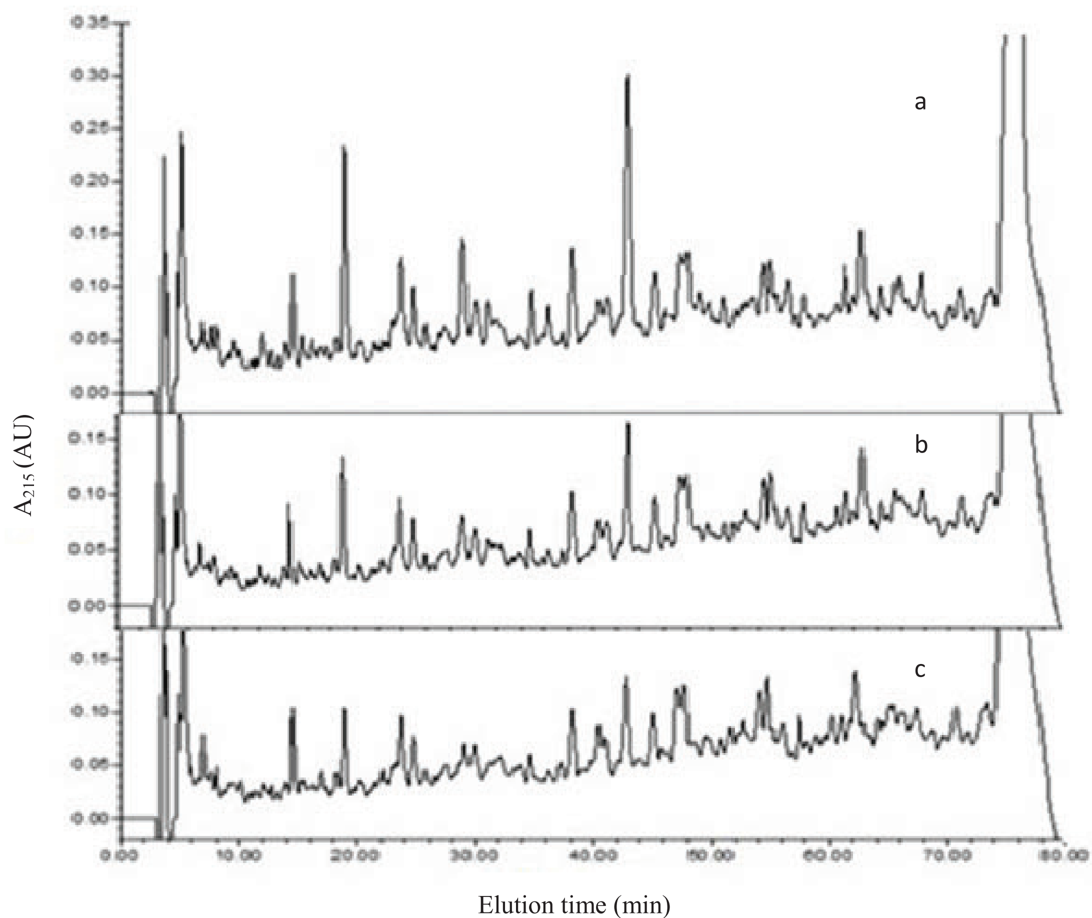

Figure 5.2 HPLC chromatograms of enzymatic hydrolysates of salmon mince with different enzyme concentrations. a) pepsin: 10,000 units/g fish protein; trypsin/chymotrypsin: 10,000 units/g fish protein; b) pepsin: 5,000 units/g fish protein; trypsin/chymotrypsin: 5,000 units/g fish protein; c) pepsin: 2,000 units/g fish protein; trypsin/chymotrypsin: 2,000 units/g fish protein. Column, Waters X-Bridge BEH 130 C_{18}; eluant A, water with 0.1% TFA; eluant B, 100% methanol with 0.1% TFA; flow rate 1 mL/min; detection at 215 nm.

5.1.5 Optimization of incubation time

Incubation time is also one of the most important factors in enzyme hydrolysis and can affect the degree of hydrolysis and therefore the bioactivities of the peptides produced. Generally speaking, properly extending the hydrolysis time can increase the peptide recovery and degree of hydrolysis (Haslaniza et al., 2010). This strategy can often be used to compensate for an intentional reduction in enzyme concentration (James et al., 2005). Nonetheless, the prolonged hydrolysis time may result in microbial proliferation and subsequently increase the cost of production. Here, the question of incubation time was addressed using ninhydrin as an indicator of the generation of free amino groups (peptides, amino acids) exposed as enzymatic hydrolysis takes place and therefore is a measure of "level of digestion".

Ninhydrin can react with primary amino groups to form a purple chromophore that absorbs strongly at 570 nm (Friedman, 2004). The intensity of the color is positively related to the number of primary amino groups (McGrath, 1971). Therefore, the stop point of the pepsin, trypsin and chymotrypsin hydrolysis can be approximately determined as the time to produce the maximum absorbance. The results are shown in Figures 5.3 and 5.4.

Figure 5.3 shows that for salmon, when the initial pepsin digestion is tested using 3 different enzyme concentrations (10,000 units/g fish protein, 5,000 units/g fish protein and 2,000 units/g fish protein), the rate of digestion appeared to increase with increasing enzyme concentration at least for the concentrations tested. The hydrolysis went on quickly for the first 9 h regardless of pepsin concentration. Subsequent hydrolysis was slow up to 24 h of incubation possibly because of the decrease in substrate over extended time periods or the inhibition by the end-products. Ideally, if the hydrolysis time is sufficient, even if the enzyme concentration is low, the hydrolysis of the same protein solution would be expected

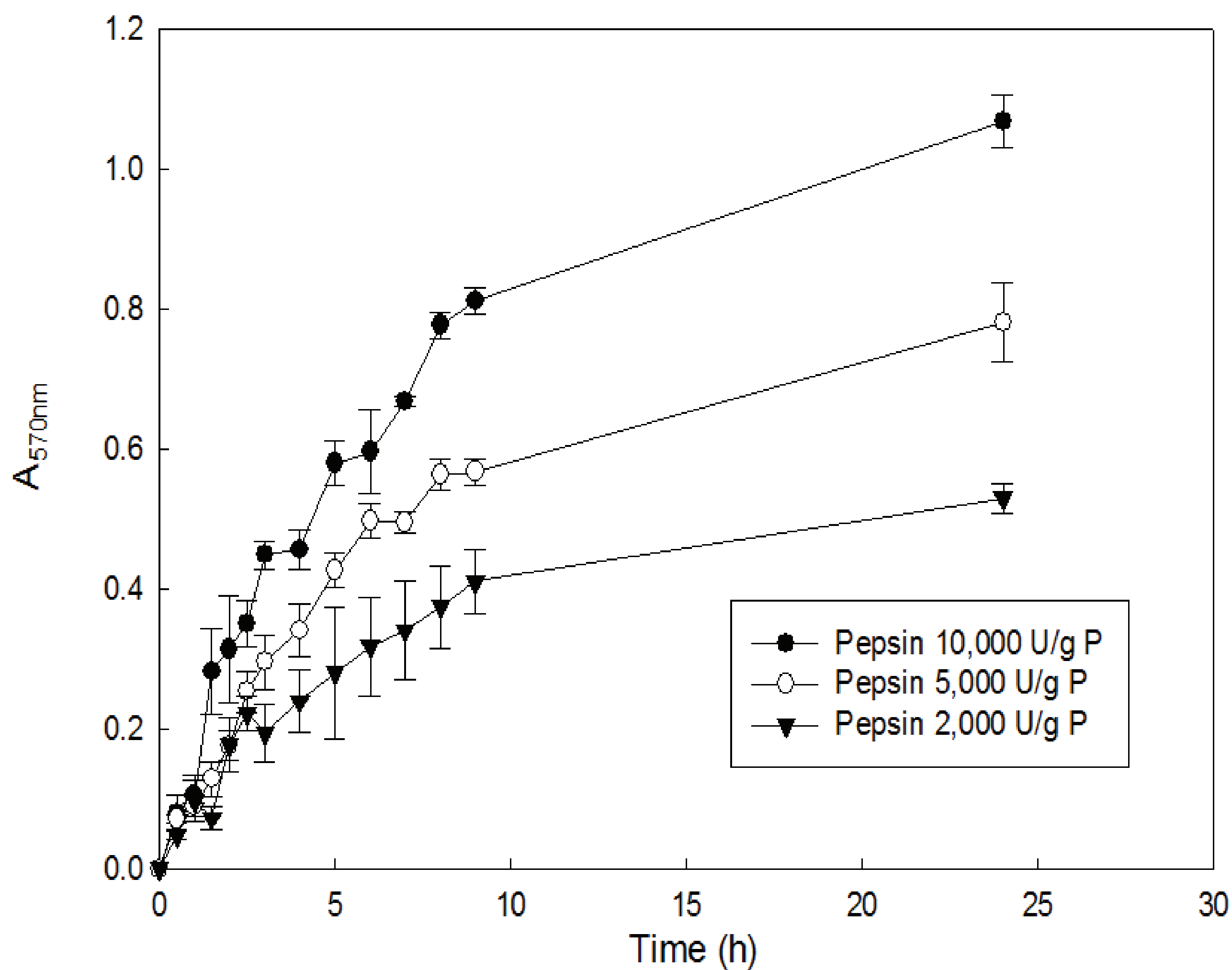

Figure 5.3 Effect of pepsin concentration on salmon protein hydrolysis. Results in figure are the mean and the standard error of the mean (SEM) of three experiments. The ninhydrin-reactive substances were measured at 570 nm to reflect the concentration of peptide released.

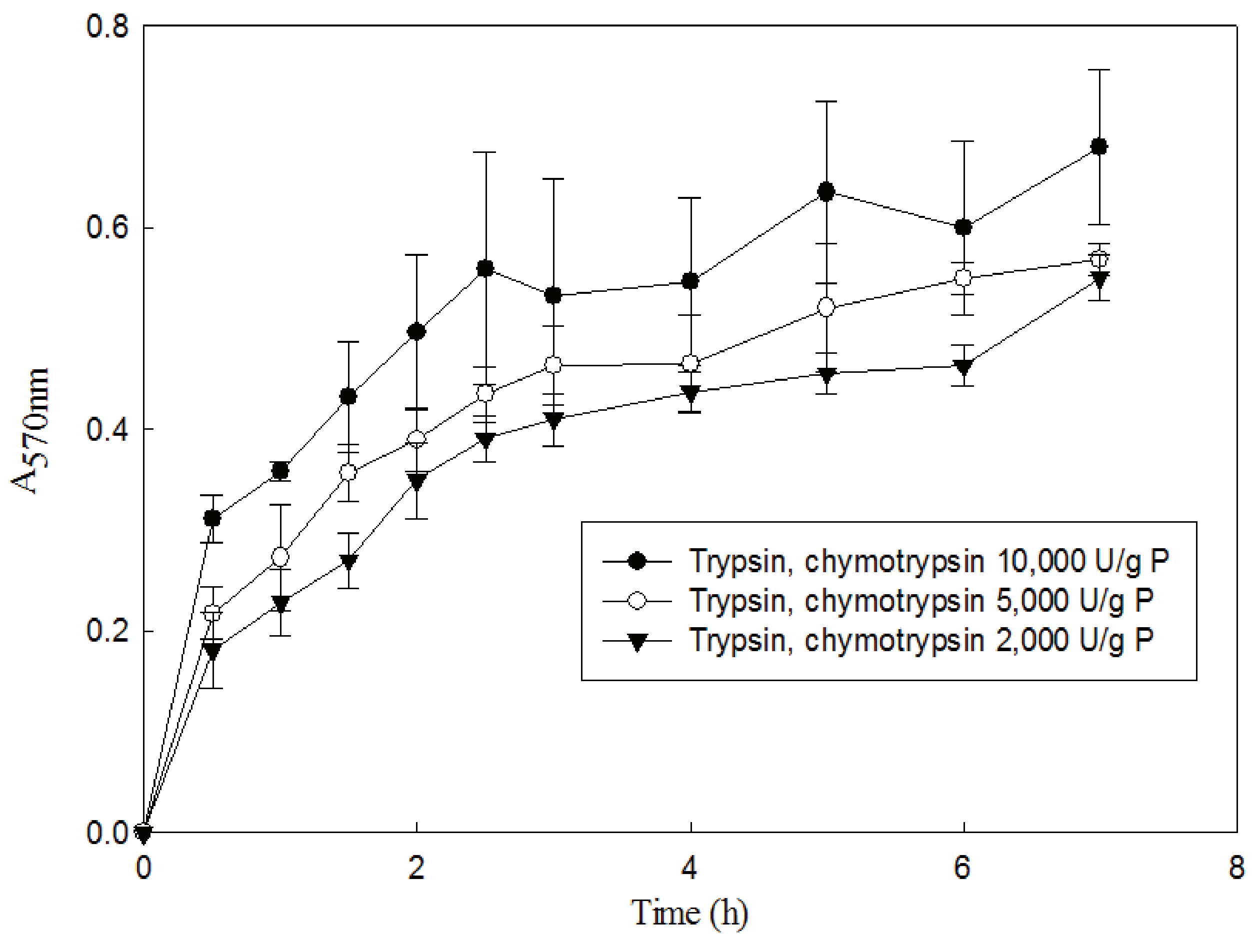

Figure 5.4 Effect of trypsin/chymotrypsin concentration on the hydrolysis of pepsin pre-digested salmon protein. Results in figure are the mean and the SEM of three experiments. The ninhydrin-reactive substances were measured at 570 nm to reflect the concentration of peptide released.

to reach the same plateau. However, Figure 5.3 indicates that even after 24 h, the enzyme hydrolysis of salmon protein with medium and low pepsin concentrations did not achieve as complete a digestion as the highest pepsin level. This is probably because of enzyme denaturation or because the incubation time was insufficient. On the other hand, extending incubation time would perhaps not be feasible due to the potential microbial problems and the increased cost. In order to determine the most suitable combination of pepsin concentration and incubation time, cost, time and hydrolysis rate per unit enzyme should be considered. It appears the lowest pepsin concentration has the highest hydrolysis rate per unit enzyme if all the pepsin digestion went on 24 h, but the highest pepsin concentration can shorten the incubation time to achieve the same digestion level as the medium and low pepsin level. A further cost/benefit analysis is required to determine which combination could produce largest benefit.

The pepsin digests produced with the highest enzyme concentration (10,000 units/g fish protein) were used as substrates for the Enzeco Chymotrypsin® 1:1 digestion and as such, a considerable amount of salmon protein had already been hydrolyzed. It appeared that varying the enzyme concentration did not result in a big difference in the trypsin and chymotrypsin hydrolysis at 37°C and pH 8.0 (Figure 5.4). The trypsin and chymotrypsin hydrolysis appears to progress rather quickly regardless of the enzyme concentration for the initial 2.5 h and then little increase for the remaining time of the experiment. Therefore, if an enzyme concentration of 10,000 units/g fish protein was chosen for the pepsin digestion, the lower enzyme concentration could be used for the trypsin and chymotrypsin hydrolysis, and 2.5 h is adequate to bring about most of the release of amino nitrogen.

5.2 CHROMATOGRAPHIC SEPARATION OF PEPTIDES FROM FISH PROTEIN HYDROLYSATES

Salmon and cod protein hydrolysates (<1 kDa) were screened in Dr Marette's laboratory at Laval University or Dr McLeod's laboratory at Dalhousie University. The active peptide mixtures were further examined as a preliminary step toward subsequent sequence analysis and identification.

5.2.1 Peptide profile of the fish protein hydrolysates (FPH)

As expected, the HPLC peptide profiles of the FPH were rather complicated. RP-HPLC was accomplished on a Waters X-Bridge BEH 130 C_{18} peptide column to give some basic information of the <1 kDa mixture in order to ascertain the numbers and relative hydrophobicity. Before running the initial chromatography, simple solubility experiments were carried out in order to determine the most suitable solvents. Twenty mg lyophilized salmon and cod protein hydrolysates (<1 kDa) were dissolved in 1 mL aliquots of dH_2O. Both salmon and cod protein hydrolysates were completely soluble in dH_2O. Then 100% acetonitrile or methanol were gradually added to these aqueous solutions, both salmon and cod protein hydrolysates were readily soluble in methanol but became insoluble in aqueous acetonitrile (AN) solutions where the $AN:H_2O > 30\%$. Thus, dH_2O was used to dissolve samples and methanol/water mixtures used for the mobile phase in HPLC.

Figures 5.5 and 5.6 show the entire peptide profiles of salmon and cod protein hydrolysates (<1 kDa). Both of them contained at least 70 peptides ranging from hydrophilic to hydrophobic. Because of the large number of peaks, it was impossible to get good baseline separation. Thus, it is necessary to do a pre-separation.

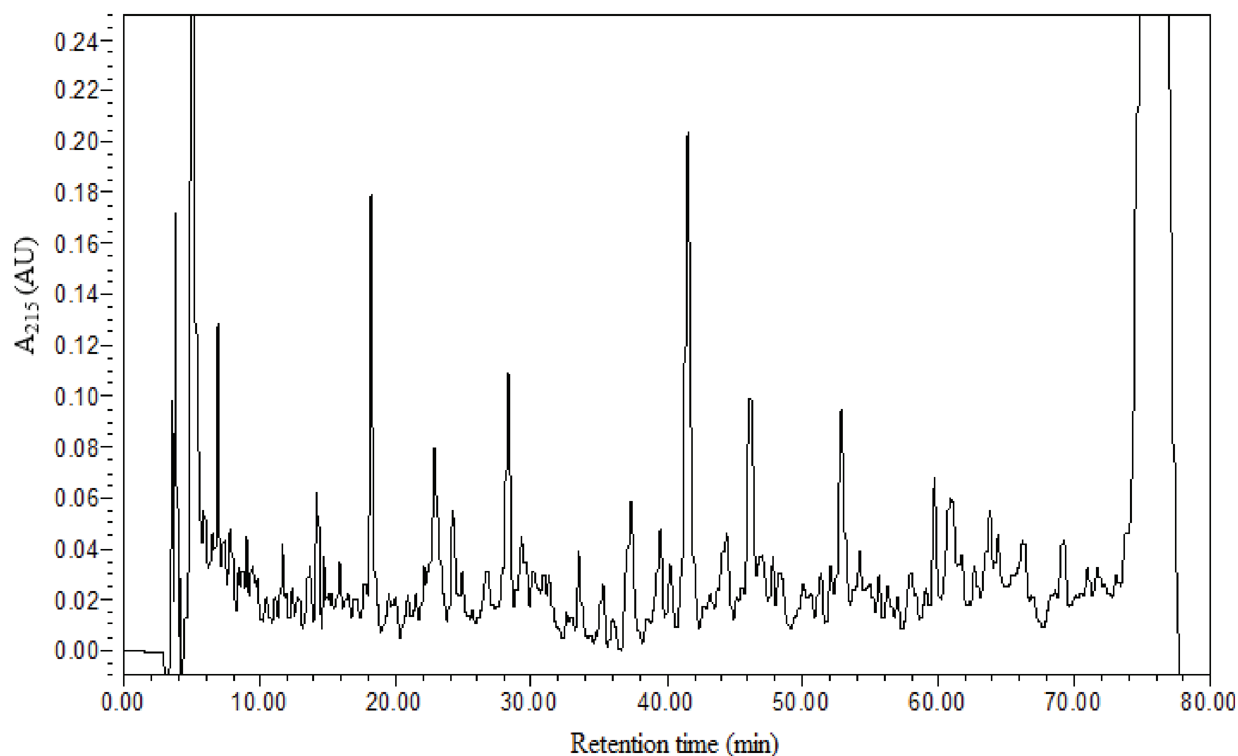

Figure 5.5 RP-HPLC peptide profile of the salmon protein hydrolysate (<1 kDa) produced by protocol 2. Column, Waters X-Bridge BEH 130 C_{18}; eluant A, water with 0.1% TFA; eluant B, 100% methanol with 0.1% TFA; flow rate 1 mL/min; detection at 215 nm.

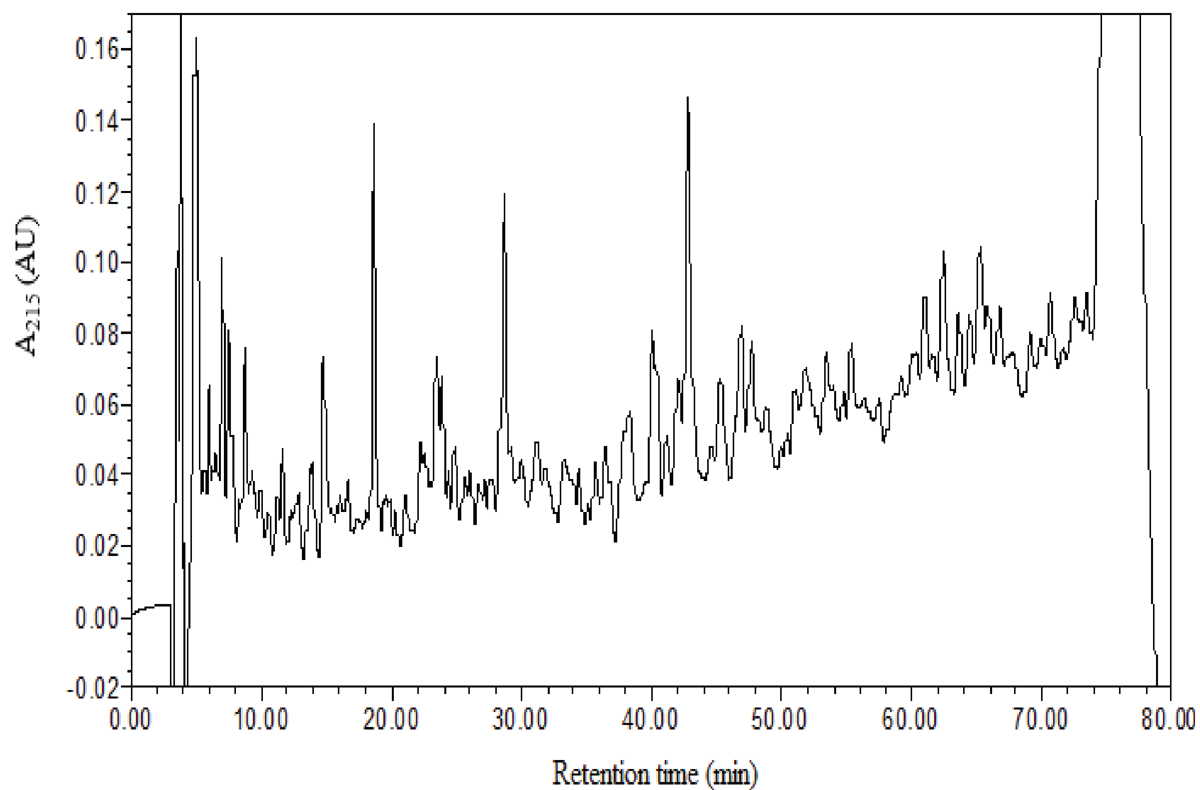

Figure 5.6 RP-HPLC peptide profile of the cod protein hydrolysate (<1 kDa) produced by protocol 3. Column, Waters X-Bridge BEH 130 C_{18}; eluant A, water with 0.1% TFA; eluant B, 100% methanol with 0.1% TFA; flow rate 1 mL/min; detection at 215 nm.

5.2.2 Size-exclusion chromatography (Gel filtration)

Size-exclusion chromatography was used to examine the apparent molecular weight (MW_{app}) distribution of freeze dried <1 kDa peptide mixture and to collect fractions for bioactivity assays.

Six calibration standards were used on the Superdex Peptide 10/300 GL column whose published separation range is 100-7000 Da (Figure 5.7). Since the molecular weight of cytochrome C is 12.5 kDa which is out of the range, this standard was used to determine the column void volume (V_o) which was 9.82 mL. Figure 5.7 also shows the relationship between log MW and elution volume (V_e) of the standards. According to the standard curve, the MW_{app} of the unknowns can be estimated using linear regression.

The size exclusion chromatogram (Figure 5.8) of salmon peptides on the Superdex Peptide column showed three major peaks and the first peak was eluted at 16.53 mL, corresponding to a MW_{app} of 1559 Da. Although this appears to be slightly higher than expected (having been recovered in the permeate of a 1000 Da molecular weight cut off (MWCO) membrane filter), one must keep in mind that the published limits for the gel filtration column were determined with standards with axial ratios of ~1.0 (spherical), and our sample is peptide mixture with different axial ratios, so it is difficult to obtain the exact molecular weight by this size exclusion column. The second and third peaks eluted at 18.05 and 19.92 mL, corresponding to MW_{app} values of 615 and 195 Da. Since the gastrointestinal absorption of peptides is restricted to amino acids, di- and tri-peptides (Pedroche et al., 2004), it is likely that fractions 2 and 3 may have higher bioactivity.

For cod peptide fractionation, two major peaks (Figure 5.9) were observed. The first was eluted at 16.25 mL while the second was eluted at 19.85 mL corresponding to MW_{app}

values of 1851 and 204 Da, respectively. Further bioactivity experiments are required for determining which fraction is more active.

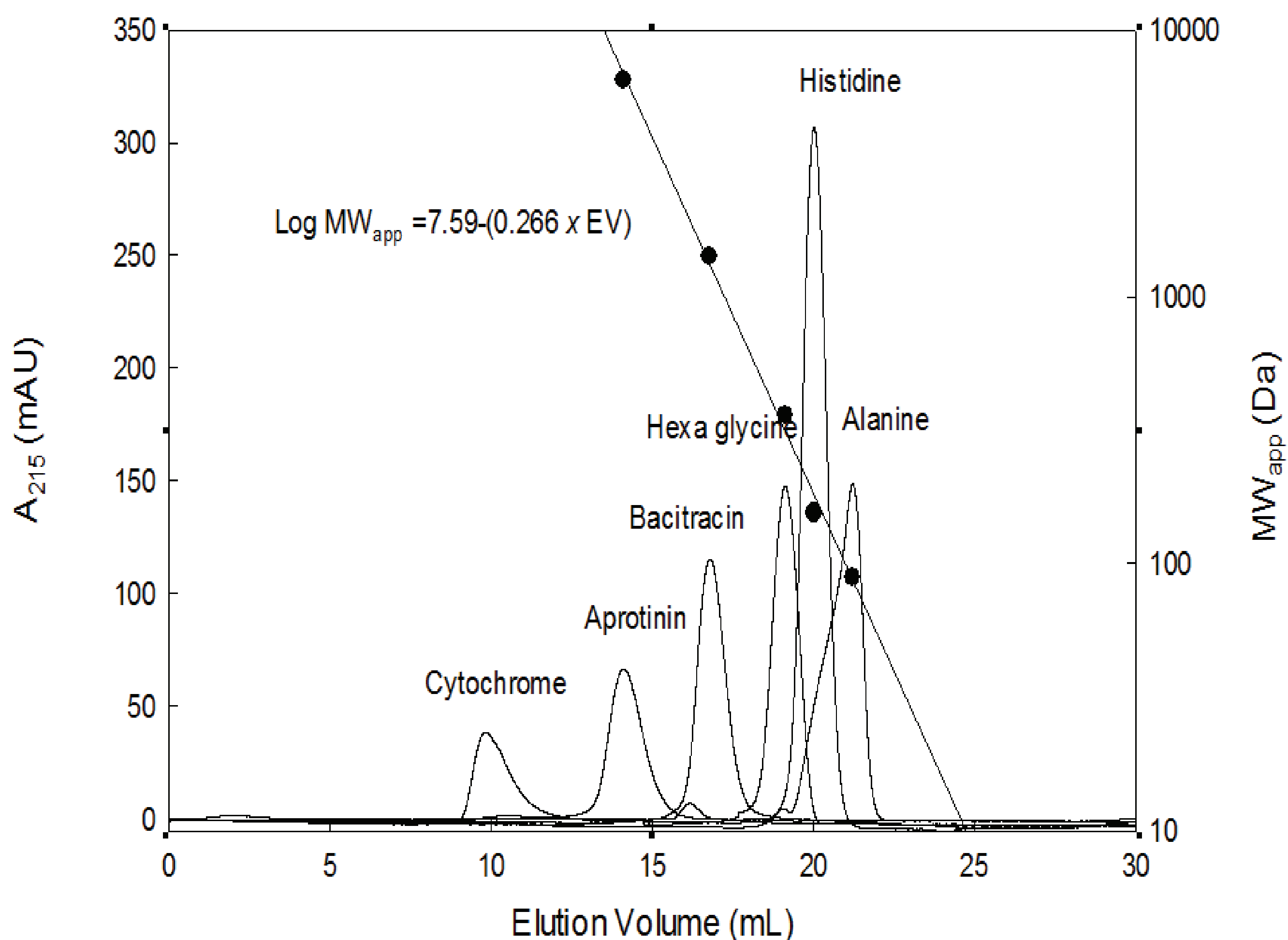

Figure 5.7 Elution profiles of the MW standards on the Superdex Peptide 10/300 GL column and the relationship between log MW and V_e of standards. Elution volume was determined at the maximum peak height of each sample. Eluant, 30% acetonitrile containing 0.1% TFA; flow rate 0.3 mL/min; detection at 215 nm. MW standard curve denoted as linear regression line.

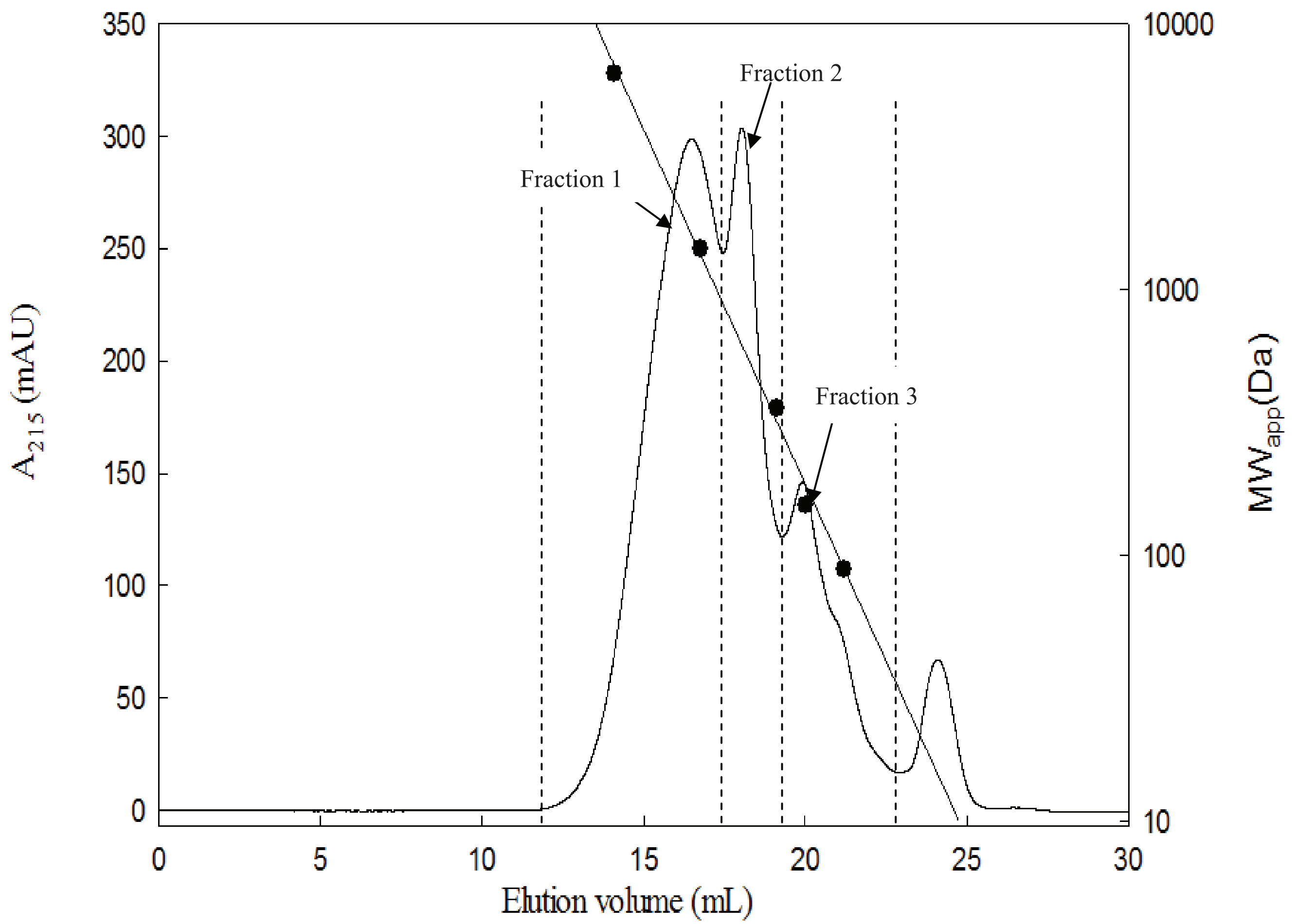

Figure 5.8 Gel filtration of the salmon protein hydrolysate (<1 kDa) produced by protocol 2 on a Superdex Peptide 10/300 GL column. Eluant, 30% acetonitrile containing 0.1% TFA; flow rate 0.3 mL/min; detection at 215 nm. MW standard curve denoted as linear regression line.

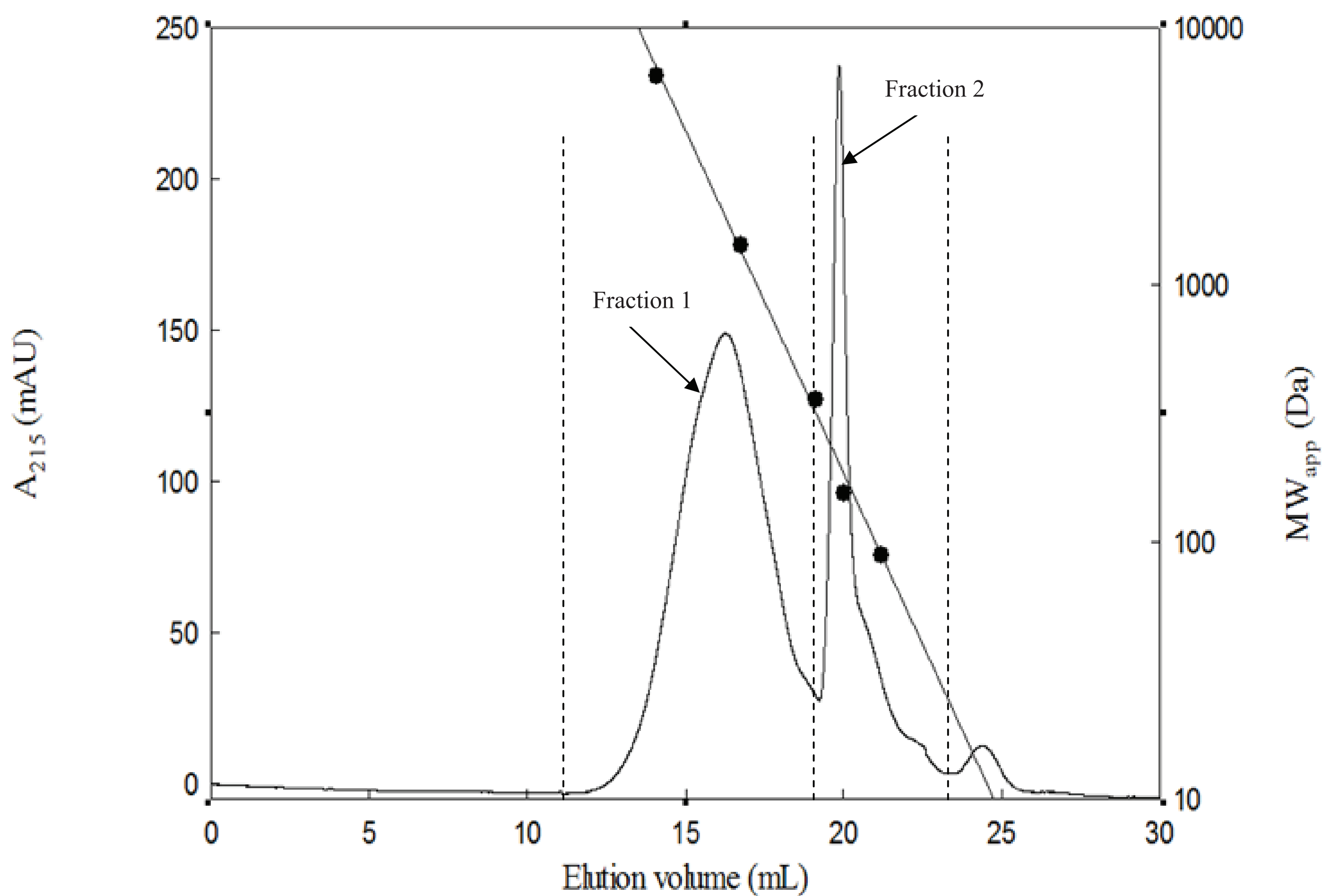

Figure 5.9 Gel filtration of the cod protein hydrolysate (<1 kDa) produced by protocol 3 on a Superdex Peptide 10/300 GL column. Eluant, 30% acetonitrile containing 0.1% TFA; flow rate 0.3 mL/min; detection at 215 nm. MW standard curve denoted as linear regression line.

5.2.3 Preparative-scale HPLC fractionation of salmon and cod protein hydrolysates

Preparative fractionation of salmon and cod protein hydrolysates produced by protocol 2 and 3, respectively, were carried out at the University of Manitoba with the Jupiter C_{12} column. The chromatographic peaks were pooled into four major fractions and freeze-dried for bioactivity tests. The preparative HPLC chromatograms are shown in Figures 5.10 and 5.11. As expected, the prep scale HPLC resolution was poor in comparison to the analytical separations shown in Figure 5.5 and 5.6. One of the possible reasons is the poorer retention ability of C_{12} column than that of C_{18} column, another important reason is the huge injection volume and sample loading (400 mg protein per injection) which would decrease the resolution of the sample.

Figures 5.10 and 5.11 show that for cod, Fraction 4 contains low peptide levels as compared to that of salmon, which suggests that salmon protein hydrolysates contain more hydrophobic peptides than cod. The activities of these fractions were tested using tissue culture assays at Laval University and were found to be devoid of activity. The reason for this may have been the result of prolonged exposure to methanol.

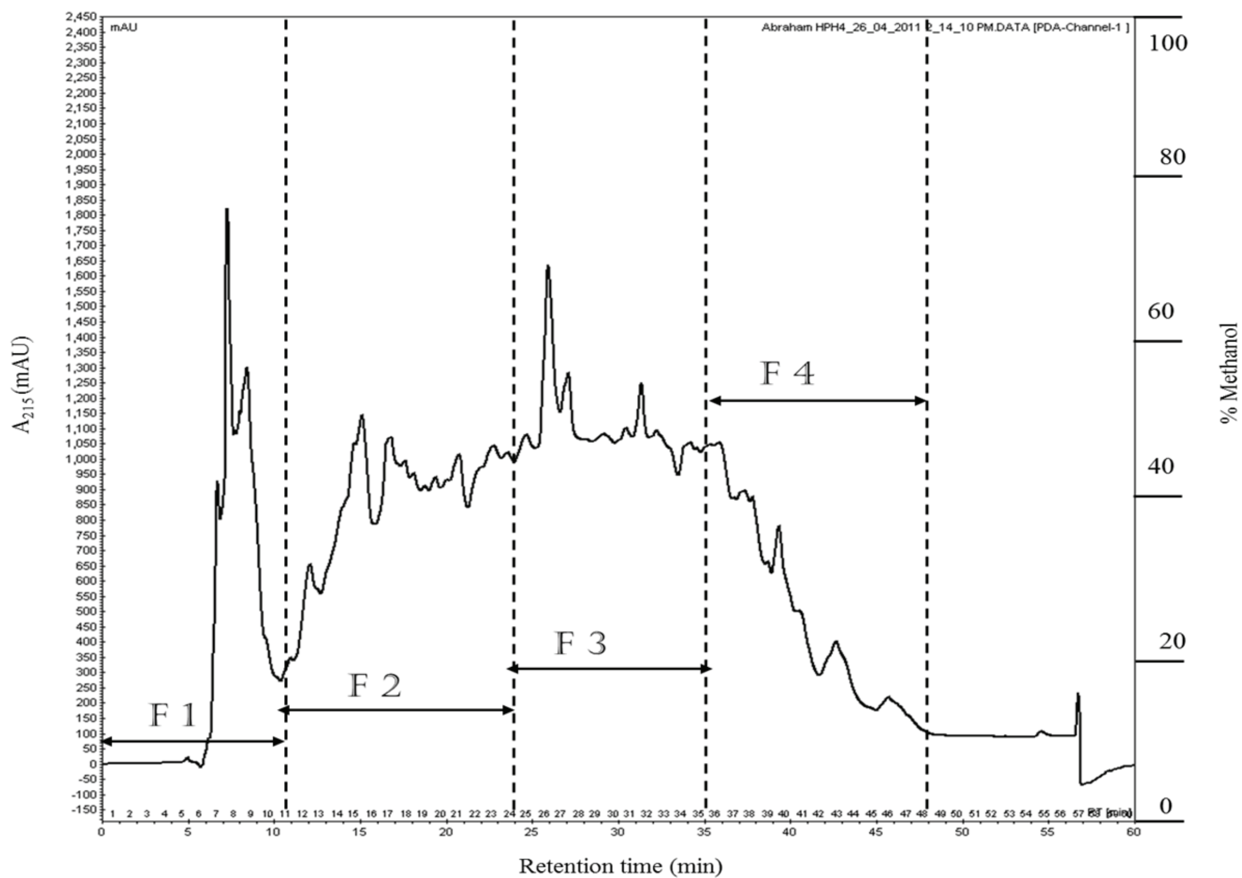

Figure 5.10 Preparative HPLC fractionation of salmon protein hydrolysates (<1 kDa) produced by protocol 2 on a Jupiter C_{12} column. Eluant A, water with 0.1% TFA; Eluant B, 100% methanol with 0.1% TFA; flow rate 10 mL/min; detection at 215 nm.

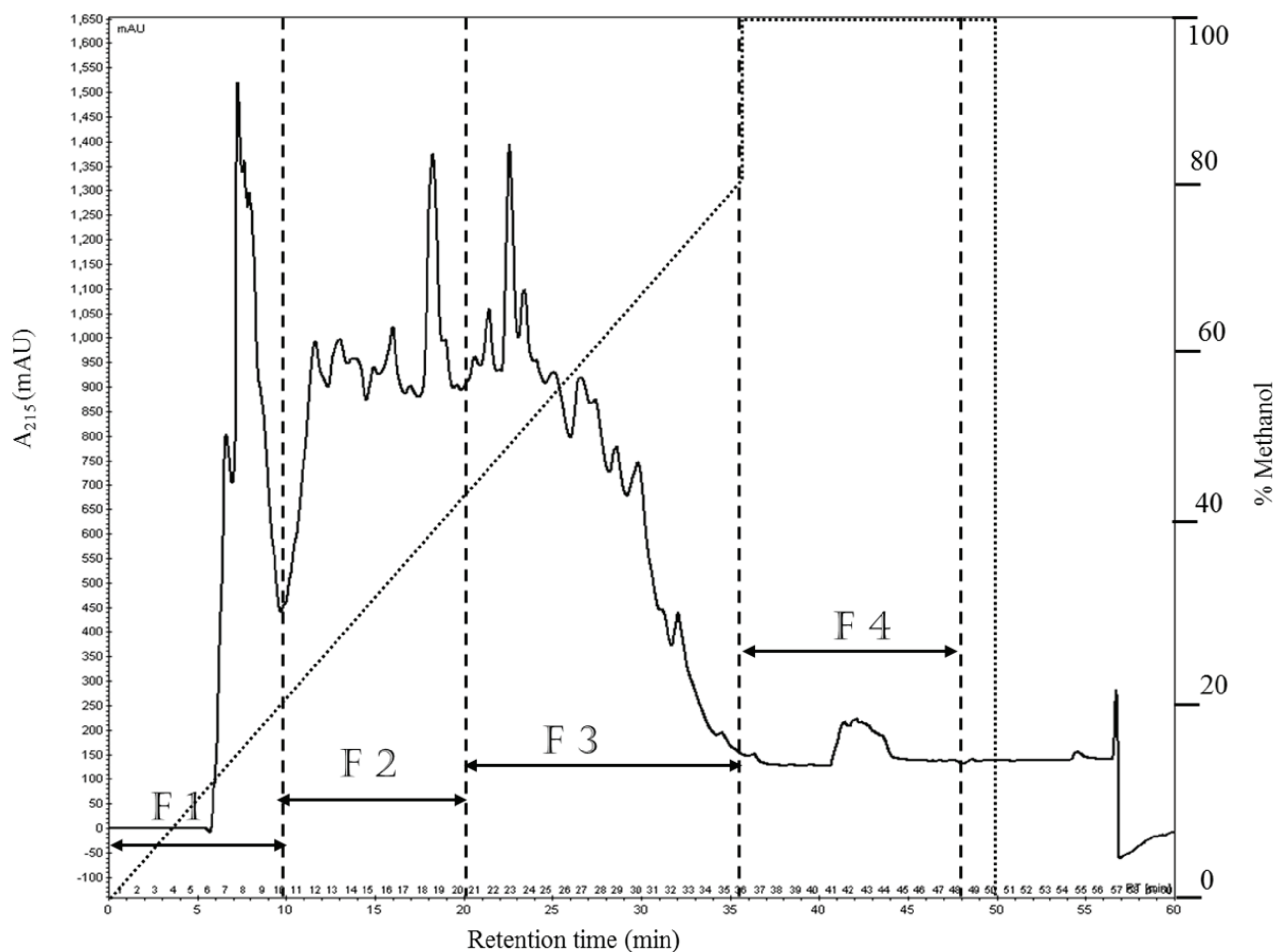

Figure 5.11 Preparative HPLC fractionation of cod protein hydrolysates (<1 kDa) produced by protocol 3 on a Jupiter C_{12} column. Eluant A, water with 0.1% TFA; Eluant B, 100% methanol with 0.1% TFA; flow rate 10 mL/min; detection at 215 nm.

5.2.4 Ion exchange chromatography

The salmon protein hydrolysate (<1 kDa) produced by protocol 2 was chromatographed on a UNOsphere^{TM} Q anion-exchange column (10×150 mm) and three fractions were obtained (Figure 5.12). Fraction 1 contained acidic peptides that were not retained by the column. Fractions 2 and 3 represented the bound peptides that were eluted by a salt gradient (0 – 1.0 M NaCl) Tris-HCl buffer (pH 8.0). Since the fractionation was carried out on a strong anion-exchange column, it indicated that fraction 1 was highly acidic even at pH 8.0, while fractions 2 and 3 were negatively-charged basic peptide mixtures, and the alkalinity of fraction 3 is evidently stronger than that of fraction 2.

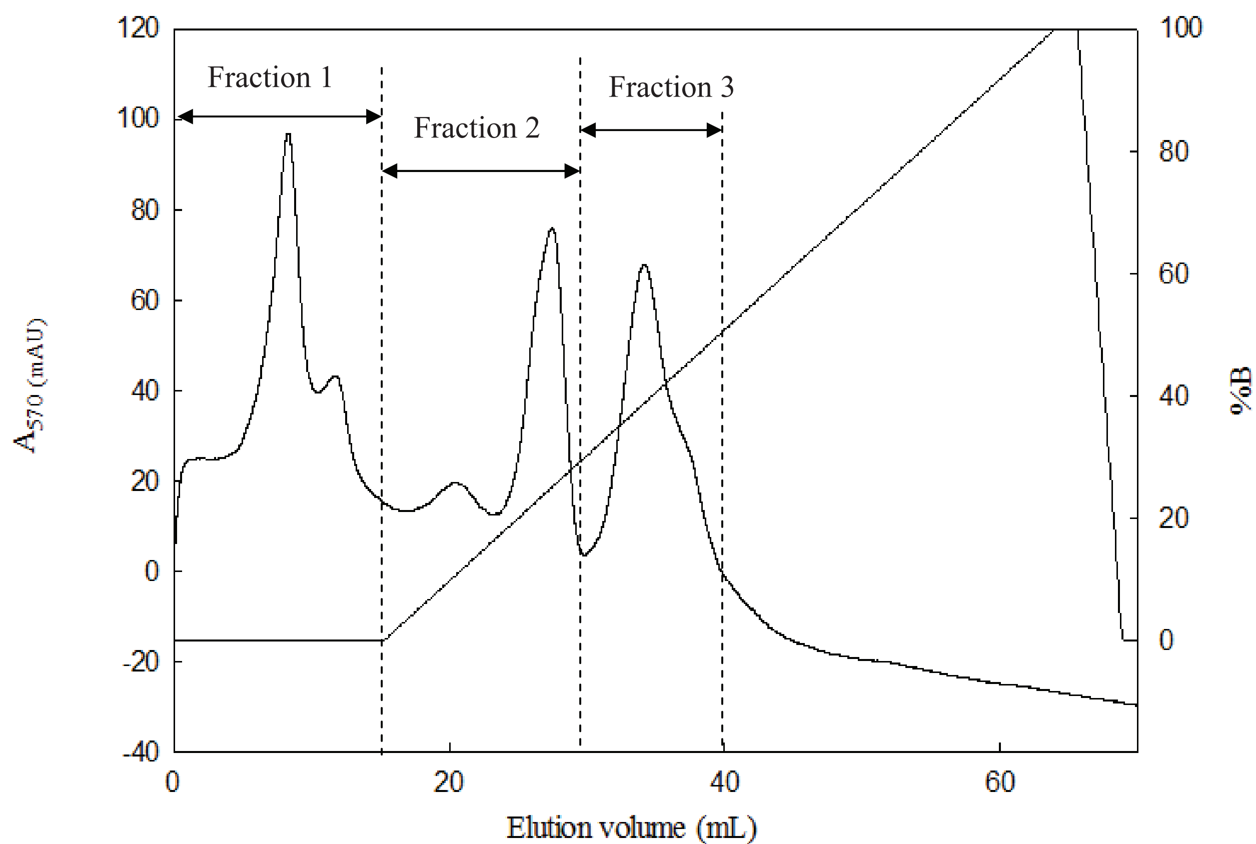

Figure 5.12 Ion exchange chromatography of salmon protein hydrolysate (<1 kDa) produced by protocol 2 on UNO sphere Q column. Eluant A, 20 mM Tris-HCl buffer (pH 8.0); elunat B, 1.0 M NaCl in 20mM Tris-HCl buffer (pH 8.0); flow rate 0.5 mL/min; detection at 215 nm.

5.3 BIOACTIVITY OF THE FISH PROTEIN HYDROLYSATE

The bioactivity tests were carried out by the Marette laboratory at Laval University and the McLeod laboratory at Dalhousie University. Dr. Marette's lab was responsible for screening peptide activities related to insulin sensitivity, insulin action on glucose metabolism in various tissues, pancreatic insulin secretion and inflammation. Dr. McLeod's lab was responsible for screening activities of peptides in 3T3-L1 pre-adipocyte culture and in hepatocyte models for changes in hepatic lipid and lipoprotein production. Brief conclusions of the results are listed below (Figures not shown here).

5.3.1 Bioactivity of original eight fish protein hydrolysates (<1 kDa)

The bioactivities of the eight FPH (<1 kDa) samples from Protocols 1, 2 and 3 (Section 4.2) were screened for glucose uptake in L6 myocytes in Marette's lab. The results showed that the pepsin hydrolysed cod (Protocol 1) can enhance basal glucose uptake (at 1 μg/mL) but not the insulin-induced glucose uptake, whereas pepsin-hydrolysed salmon (Protocol 1) had no significant biological effects. The pancreatin hydrolysate from either cod or salmon protein (Protocol 1) increased the basal glucose uptake (at 1 mg/mL), but there were no effects observed in pancreatin-derived salmon/cod protein hydrolysate on insulin-induced glucose uptake. The results also showed that cod and salmon proteins extracted in 1.0 M NaOH and hydrolyzed with pepsin, trypsin and chymotrypsin (Protocol 2) were both effective on insulin-induced glucose uptake, whereas cod and salmon proteins dispersed in water and then digested with pepsin, trypsin and chymotrypsin (Protocol 3) increased both basal and insulin-induced glucose uptake, and the salmon peptides were more effective than cod peptides.

5.3.2 Bioactivity of selected two fish protein hydrolysates (<1 kDa)

Based upon the preliminary results, salmon protein hydrolysate (<1 kDa) prepared from alkali-solubilisation and then sequential hydrolysis with pepsin/trypsin/chymotrypsin were

used for further fractionation and screening. Also, water-dispersed cod proteins digested with pepsin/trypsin/chymotrypsin were further screened using *in vitro* models.

5.3.2.1 Glucose production assay

Hepatic gluconeogenesis is known to be particularly elevated in conditions of insulin resistance and diabetes and therefore any decrease in hepatic gluconeogenesis would signify an improvement in the metabolic condition. Hepatic glucose production (HGP) was evaluated in FAO rat hepatocytes (a differentiated rat liver cell line derived from rat H4IIE hepatoma cells) in both the basal and insulin-suppressed conditions. Hepatocytes treated with salmon protein hydrolysate (<1 kDa) showed an improvement (reduction in hepatic glucose production) in both the basal and the insulin-suppressed conditions while cod protein hydrolysate (<1 kDa) showed a similar trend for improvement.

5.3.2.2 Inflammation modulation

Nitric oxide (NO) production was used as an index of inducible NO synthetase activation and inflammation in the macrophage. Salmon protein hydrolysate (<1 kDa) treatment at either 1 ng or 1µg/mL significantly reduced NO production by almost 40% compared to an LPS control. To a lesser extent cod peptides at a concentration of 1µg/mL were also able to reduce NO production.

5.3.2.3 Adiponectin metabolism modulation

The secretion of adiponectin from differentiated mouse 3T3-L1 pre-adipocytes were studied in order to examine the ability of fish protein hydrolysates to promote adiponectin production in adipose tissue. Adipocytes were treated with up to 1 mg/mL of salmon and cod protein hydrolysates (<1 kDa). The results shown that salmon protein hydrolysate stimulated secretion of adiponectin in a dose-dependent manner, whereas cod peptides did not. Neither the salmon nor the cod protein hydrolysates increased the cellular adipoenctin mass, nor did the salmon protein hydrolysates affect the multimer distribution of secreted

adiponectin.

Taken together these results appear to indicate that the salmon protein hydrolysate of < 1 kDa is the most biologically active and warranted the initiation of *in vivo* experiments in a model of diet induced obesity

5.3.2.4 *In vivo* mouse feeding trials

In a first study LDLr-/-/ApoB100/100 mice, a well-established model of atherosclerosis were fed either a standard low fat chow diet, or a high fat high sucrose (HFHS) diet to promote the development of obesity and insulin resistance. Other groups of HFHS-fed LDLr-/-/ApoB100/100 mice were also supplemented with either 10% salmon protein hydrolysate (<1 kDa), an omega-3-rich fish oil, or both for 6 months. Adding salmon protein hydrolysate (<1 kDa) to the HFHS diet significantly reduced fasting hyperinsulinemia and glucose intolerance after only 3 months of treatment. When used in combination, both salmon protein hydrolysate and fish oil almost fully protected HFHS-fed animals from glucose intolerance, without any significant effects on weight gain or adipose tissue mass. Pyruvate tolerance tests also revealed that salmon protein hydrolysate dramatically reduced hepatic glucose production from gluconeogenesis in these mice, suggesting that the liver is a major site of salmon protein hydrolysate action.

5.3.3 Bioactivity of peptide fractions from preparative HPLC fractionation

5.3.3.1 Bioactivity of fish protein hydrolysates (<1 kDa) fractions separated on a Jupiter C_{12} column

The bioactivities of salmon and cod peptide fractions separated by Jupiter C_{12} column were screened for glucose uptake in L6 myocytes and NO production in macrophages. The results show that both salmon and cod protein hydrolysates separated on the basis of hydrophobicity didn’t distinguish a more bioactive fraction. Indeed, fractions fractionated by hydrophobicity appear to inhibit glucose uptake in the insulin- stimulated state and only

minimally reduce NO production. There were no differences among these fractions. Since the unfractionated salmon and cod protein hydrolysates (<1 kDa) can significantly increase glucose uptake in L6 myocytes and reduce NO production in macrophage, the loss of activity when separated by hydrophobicity indicated that the active components of FPH may be a combination of peptides rather than one single peptide or perhaps this separation method somehow adversely affected the bioactivities of the FPHs.

5.3.3.2 Bioactivity of Superdex Peptide 10/300 GL column separated fractions of fish protein hydrolysates (<1 kDa)

The bioactivities of salmon and cod peptide fractions separated on a Superdex Peptide 10/300 GL column were screened. The smaller cod peptide fractions eluting later in the chromatograms showed a greater reduction in the NO production than the original cod protein hydrolysate (<1 kDa). Further screening tests will be conducted by Dr. Marette's laboratory, and the bioactivities of salmon and cod peptide fractions separated by ion-exchange column will also be screened in order to find out the active peptides for the further identification and amino acid sequence analysis.

CHAPTER 6. CONCLUSIONS

6.1 CONCLUSIONS

Several methods were used to help solubilise or disperse mechanically-deboned salmon and cod muscle proteins and subsequent digestion with assorted proteolytic enzymes. Of the methods attempted, a protocol involving alkali solubilisation as well as isoelectric precipitation, followed by sequential hydrolysis with pepsin, trypsin and chymotrypsin was developed for the production of bioactive peptides from Atlantic salmon tissue. A highly active peptide mixture was produced by segregating the < 1 kDa mixture from the higher molecular weight material using preparative ultrafiltration. A second-protocol using water dispersion was developed for cod. The peptide mixtures generated were tested for bioactivity by *in vitro* and *in vivo* methods. Salmon and cod protein hydrolysates (<1 kDa) from fish mince had overall nitrogen recoveries of 16.9% and 40.1%, respectively from the minced starting materials. Although losses of yield were high during the ultrafiltration process, this is not necessarily a bad thing since ultrafiltration resulted in considerable concentration of the bioactive components in the digests.

The batch size for producing salmon protein hydrolysate (<1 kDa) was scaled up to a maximum of 12.5 kg and showed good reproducibility. Substitution of trypsin and chymotrypsin with a lower cost, commercially available blend did not result in a significant change in the final composition of the FPH.

The salmon and cod protein hydrolysates (<1 kDa) were shown to have the ability to increase glucose uptake in L6 myocytes, reduce glucose production in FAO rat hepatocytes and decrease NO production in macrophages. The *in vivo* mouse feeding trials also demonstrated that salmon protein hydrolysate (<1 kDa) can significantly reduce fasting

hyperinsulinemia and glucose intolerance in the HFHS fed mice.

6.2 FUTURE WORK

Enzymatic hydrolysis of fish protein using pepsin, trypsin and chymotrypsin appears to be an effective method to produce and to concentrate bioactive peptides. The <1 kDa peptide mixture from salmon displayed antidiabetic properties. Future work should focus on the identification of the active peptide(s) responsible for the remarkable ability to modulate the various biomarkers associated with type 2 diabetes and its associated metabolic syndrome. Therefore, further separation and bioactivity screening experiments are required. Also, the mode (s) of action at the molecular level will be determined. In addition, more work has to be done on the fractionation of FPH including separation on the basis of charge, molecular size and possible affinity for selected ligands. This is a complex and time-consuming process and was not within the scope of this thesis.

The final goal of this project is recovering of anti-diabetic peptides from salmon or cod waste on a commercial-scale. It is for this reason that experiments were carried out to minimize production costs as well as reducing production time. A commercially immobilized enzyme or membrane bioreactor system should be developed. In addition, a commercial scale process for the rapid and complete removal of salt (produced in the current manufacturing protocol) must be developed or alternatively, a new process could be developed that does not require the use of alkali solubilisation and isoelectric precipitation. In the future, a reproducibility method of analysis will be developed so that these peptides can be determined in complex food mixture.

REFERENCES

Aberoumand, A. (2011). Proximate composition and energy values of canned tuna fish obtained from Iran. *Middle-East Journal of Scientific Research. 9* (4), 442-446.

American Diabetes association. (2010). *Hyperglycemia (High blood glucose).*Retrieved from http://www.diabetes.org/living-with-diabetes/treatment-and-care/blood-glucose-control/hyperglycemia.html.

Aguilar, M. I. (Ed.). (2004). *HPLC of peptides and proteins: methods and protocols*. New Jersey: Humana Press.

Aguilar, M. I., & Hearn, M. T. W. (1996). High resolution reversed phase high performance liquid chromatography of peptides and proteins. *Methods in Enzymology*, 3-26.

Allen, G. (1981). Sequencing of proteins and peptides. *Laboratory Techniques in Biochemistry and Molecular Biology*, 9, 73-135.

Amersham Biosciences. (2002). *Gel filtration: principles and methods.* New Jersey: GE healthcare.

Amersham Biosciences. (2002). *Ion exchange chromatography: principles and methods.* New Jersey: GE healthcare.

Aoyama, T., Fukui, K., Nakamori, T., Hashimoto, Y., Yamamoto, T., & Takamatsu, K. (2000). Effect of soy and milk whey protein isolates and their hydrolysates on weight reduction in genetically obese mice. *Bioscience Biotechnology and Biochemistr*y, *64*, 2594-2600.

Aoyama, T., Fukui, K., Takamatsu, K., Hashimoto, Y., & Yamamoto, T. (2000). Soy protein isolate and its hydrolysate reduce body fat of dietary obese rats and genetically obese mice (*yellow KK*). *Nutrition, 16,* 349-354.

Aquaculture Statistics. (2009). Statistics Canada.

Arvanitoyannis, I. S., Kassaveti, A. (2008). Fish waste Management: Treatment Methods and Potential Uses of Treated Waste. Arvanitoyannis, I. S. (Ed.), *Waste Management for the Food Industries* (pp. 861-937). London: Elsevier.

Azuma, B., Machida, K., Saeki, Y., Kanamoto, R., & Iwami, K. (2000). Preventive effect of soybean resistant proteins against experimental tumorigenesis in rat colon. *Journal of Nutritional Science and Vitaminology, 16*, 349-354.

Bailey, C. J. (2000). Potential new treatments for type 2 diabetes. *Trends in Pharmacological Sciences, 21*, 259-266.

Baker, R. W., Koros, W. J., Cussler, E. L., Riley, R. L., Eykamp, W., & Strathmann, H. (1991). *Membrane Separation Systems: Recent Developments and Future Directions.* USA: Noyes Data Corporation.

Batista, I. (2000). Recovery of proteins from fish waste products by alkaline extraction. *European Food Research and Technology*, *210* (2), 84-89.

Bazinet, L., & Firdaous, L. (2009). Membrane processes and devices for separation of bioactive peptides. *Recent Patents on Biotechnology*, *3*, 61-72.

Berg, J. M., Tymoczko, J. L., & Stryer, L. (2007). *Biochemistry* (6th ed.). New York: W. H. Freeman.

Biotech, A. P. (2000). *Hydrophobic Interaction Chromatography: principles and methods.* New Jersey: GE healthcare.

Bougatel, A., Arroume, N. N., Manni, L., Ravallec, R., Barkia, A., Guillochon, D., & Nasri, M. (2010). Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (*Sardinella aurita*) by-products proteins. *Food Chemistry*, *118* (3), 559-565.

Brady, S. T. (Ed.). (2011). *Basic Neurochemistry: principles of molecular, cellular, and medical neurobiology* (8th ed.). Waltham: Elsevier Inc.

Byun, H. G., & Kim, S. K. (2001). Purification and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides from Alaska pollack (*Theragra chalcogramma*) skin. *Process Biochemistry*, *36*, 1155-1162.

Cabot, S., & Jasinska, M. (2007). *Diabetes Type 2 - You can reverse it naturally, lose weight and restore your health.* Glendale: SCB International Inc.

Campbell, R. K., & White, J. R. (2002). Insulin therapy in type 2 diabetes. *Journal of the American Pharmacists Association*, *42* (4), 602-611.

Canadian Diabetes Association. (2010). *Canada at the Tipping point-charting a new path*. Retrieved from http://www. diabetes.ca/documents/get-involved/ WEB_Eng.CDA_ Report.pdf.

Chen, H. M, Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998). Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *Journal of Agricultural and Food Chemistry, 46*, 49-53.

Choudhary, P. (2004). Review of dietary recommendations for diabetes mellitus. *Diabetes Research and Clinical Practice, 65S*, S9-S15.

Chun, B. S., Kishimura, H., Nalinanon, S., Klomklao, S., & Benjakul, S. (2011). Mackerel trypsin purified from defatted viscera by supercritical carbon dioxide. *Journal of Amino Acid*.

Cohen, S. A. (2008). *Multidimensional Liquid Chromatography (MDLC) is a very powerful separation technique for analyzing exceptionally.* New Jersey: John Wiley & Sons, Inc.

Colberge, S. R. (2006). The impact of exercise on insulin action in type 2 diabetes mellitus: Relationship to prevention and control. *Insulin, 1* (3), 85-98.

Conlon, J. M. (2007). Purification of naturally occurring peptides by reversed-phase HPLC. *Protocol, 2* (1), 191-198.

Daffre, S., Bulet, P., Spisni, A., Sabatier, L. E., Rodrigues, E. G., & Travassos, L. R. (2008). Bioactive natural peptides. *Studies in Natural Products Chemistry*. *35*, 597-691.

DeFronzo, R. (1997). Pathogenesis of type 2 diabetes: metabolic and molecular implications for identifying diabetes genes. *Diabetes Reviews*. 177-269.

Enyard, C. C. (2008). Sigma's Non-specific protease activity assay-casein as a substrate. *Journal of Visualized Experiment.19*, e889.

Erdmann, K., Belinda, W. Y., & Cheung, H. S. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *Journal of Nutritional Biochemistry, 19*, 643-654.

Erdmann, K., Cheung, B. W. Y., & Schroder, H. (2007). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *The Journal of Nutritional Biochemistry*, *19* (10), 643-654.

Fahmi, A., Morimura, S., Guo, H. C., Shigematsu, T., & Kida, K. (2004). Production of angiotensin I converting enzyme inhibitory peptides from sea bream scales. *Process Biochemistry, 39*, 1195-1200.

Feng, X., Liu, T., Wang, F., Cao, R., Zhou, B., Zhang, Y., Mao, X., … Zhang, H. (2010). Isolation, antiproliferation on tumor cell and immunomodulatory activity of BSP-I, a novel bursal peptide from chicken humoral immune system. *Peptides, 32* (6), 1103-1109.

Foh, M. B. K., Kamara, M. T., Amadou, I., Foh, B. M., & Xia, W. S. (2011). Chemical and physicochemical properties of Tilapia (*Oreochromis niloticus*) fish protein hydrolysate and concentrate. *International Journal of Biological Chemistry, 5* (1), 21-36.

Fountoulakis, M., & Lahm, H. W. (1998). Hydrolysis and amino acid composition analysis of proteins. *Journal of Chromatography A, 826*, 109-134.

Fox. C., & Kilvert. A. (2007). *Type 2 Diabetes.* London: Class publishing.

Friedman, M. (2004). Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences. *Journal of Agricultural and Food Chemistry, 52*, 385-406.

Fritz, T., Wandell, P., Aberg, H., & Engfeldt, P. (2005). Walking for exercise – does three times per week influence risk factors in type 2 diabetes? *Diabetes Research and Clinical Practice, 71* (1), 21-27.

Galvez, A. F., Chen, N., Macasieb, J., & de Lumen, B.O. (2001). Chemopreventive property of a soybean peptide *(lunasin*) that binds to deacetylated histones and inhibits acetylation. *Cancer Research, 61*, 7473-7478.

Garrett, R., & Grisham, C. M. (2005). *Biochemistry.* Belmont: David Harris.

Gildberg, A., Bϕgwald, J., Johansen, A., & Stenberg, E. (1996). Isolation of acid peptide fractions from a fish protein hydrolysate with strong stimulatory effect on Atlantic salmon (*Salmo salar*) head kidney. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 114* (1), 97-101.

Gill, T. (2000). Waste from processing aquatic animals and animal products: implications on aquatic animal pathogen. *FAO Fisheries Circular, 956*, 26.

Giménez, B., Alemán, A., Montero, P., & Gómez-Guillén, M. C. (2009). Antioxidant and functional properties of gelatin hydrolysates obtained from skin of sole and squid. *Food Chemistry, 114*, 976-983.

Glaser, B., & Cerasi, E. (1999). Early intensive insulin treatment for induction of long-term glycaemic control in type 2 diabetes. *Diabetes, Obesity and Metabolism*, 67-74.

GmbH, T. B. (n.d.). *Principle of hydrophobic interaction chromatography*. Retrieved from http://www.separations.eu.tosohbioscience.com/ServiceSupport/ Tech Support/Resource Center/Principles of Chromatography/Hydrophobic Interaction/

Guérard, F., Decourcelle, N., Sabourin, C., Floch-Laizet, C., Le Grel, L., … Bourseau, P. (2010). Recent developments of marine ingredients for food and nutraceutical applications: A review. *Journal des Sciences Halieutiques et Aquatiques, 2*, 21-27.

Guérard, F., Dufosse, L., Broise, D. D., & Binet, A. (2001). Enzymatic hydrolysis of proteins from yellowfin tuna (*Thunnus albacares*) wastes using Alcalase. *Journal of Molecular Catalysis B: Enzymatic, 11* (4-6), 1051-1059.

Haffner, S. (1998). Epidemiology of type 2 diabetes: Risk Factors. *Diabetes care*, C3.

Hames, B. D. (1998). *Gel electrophoresis of protein: a practical approach.* New York: Oxford University Press.

Harnedy, P. A., & FitzGerald, R. J. (2011). Bioactive proteins, peptides, and amino acids from macroalgae. *Journal of Phycology, 47* (2), 218-232.

Harnedy, P. A.,& Fitzgerald, R. J. (2011). Bioactive peptides from marine processing waste. *Journal of Functional Food*, *4*, 6-24.

Hartmann, R., & Meisel, H. (2007). Food-derived peptides with biological activity: from research to food applications. *Current Opinion in Biotechnology, 18*, 163-169.

Haslaniza, H., Maskat, M. Y., Wan Aida, W. M., & Mamot, S. (2010). The effects of enzyme concentration, temperature and incubation time on nitrogen content and degree of hydrolysis of protein precipitate from cockle (*Andara granosa*) meat wash water. *International Food Research Journal*, *17*, 147-152.

Hayer, M., Stanton, C., Fitzgenrald, G. F., & Ross, R. P. (2007). Putting microbes to work: Dairy fermentation, cell factories and bioactive peptides. Part II: Bioactive peptide functions. *Biotechnology Journal, 2*, 435-449.

Herpandi, N. H., Rosma, A., & Wan Nadiah, W. A. (2011). The Tuna Fishing Industry: A New Outlook on Fish Protein Hydrolysates. *Comprehensive Reviews in Food Science and Food Safety, 10*, 195-207.

Holownia, T. (2008). Production of protein hydrolysates in an enzymatic membrane reactor. *Biochemical Engineering Journal, 39*, 221-229.

Hosomi, R., Fukao, M., Fukunaga, K., Okuno, M., Yagita, R. Kanda, S., …Yoshida, M. (2010). Effect of fish protein and peptides on lipid absorption in rats. *Nutrients Research*, *27*, 21-27.

Hsu, K. C. (2010). Purification of antioxidative peptides prepared from enzymatic hydrolysates of tuna dark muscle by-product. *Food Chemistry*, *122* (1), 42-48.

Hsu, K. C., Li-Chan, E. Y., & Jao, C. L. (2011). Antiproliferative activity of peptides prepared from enzymatic hydrolysates of tuna dark muscle on human breast cancer cell line MCF-7. *Food Chemistry, 126*, 617-622.

Huang, F. J., & Wu, W. T. (2010). Purification and characterization of a new peptide (s-8300) from shark liver. *Journal of Food Biochemistry. 34* (5), 962-970.

Hui, Y. H. (2006). *Food Biochemistry and Food Processing.* Iowa: Blackwell Publishing Professional.

Idler, D. R., Bazar, L. S., & Hwang, S. J. (1975). Fish Gonadotropin(s). II. Isolation of Gonadotropin(s) from Chum Salmon Pituitary Glands Using Affinity Chromatography. *Endocrine Research, 2* (3), 215-235.

Izadpanah A., & Gallo, R. L. (2005). Antimicrobial peptides. *Journal of the American Academy of Dermatology*, *52* (3), 381-390.

Jain, S., & Saraf, S. (2008). Type 2 diabetes mellitus – Its global prevalence and therapeutic strategies. *Diabetes & Metabolic Syndrome: Clinical Research and Review, 4*, 48-56.

James, I. T., Philip, B. G., & Sheila, A. B. (2005). Optimization of conditions for the enzymatic hydrolysis of phytoestrogen conjugates in urine and plasma. *Analytical Biochemistry*, *341*, 220-229.

Jang, H., Hyeon, J., Won, C., Lee, H., Shin Chang, S., Park, C., & Kang, J. (2010). In vivo and in vitro application of black soybean peptides in the amelioration of endoplasmic reticulum stress and improvement of insulin resistance. *Life Science, 86*, 267-274.

Je, J. Y., Park, P. J., & Kim, S. K. (2005). Antioxidant activity of a peptide isolated from Alaska pollack (*Theragra chalcogramma)* frame protein hydrolysate. *Food Research International, 38*, 45-50.

Je, J. Y., Park, P. J., Kwon, J. Y., & Kim, S. K. (2004). A novel angiotensin I converting enzyme inhibitory peptide from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. *Journal of Agricultural and Food Chemistry, 52*, 7842-7845.

Je, J. Y., Qian, Z. J., Byun, H. G., & Kim, S. K. (2007). Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis. *Process Biochemistry, 42*, 840-846.

Jeon, Y. J., Byun, H. G., & Kim, S. K. (1999). Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. *Process Biochemistry, 35*, 471-478.

Jeong, H. J., Lam, Y., & de Lumen, B. O. (2002). Barley lunasin suppresses ras-induced colony formation and inhibits core histone acetylation in mammalian cells. *Journal of Agricultural and Food Chemistry, 50*, 5903-5908.

Jun, S. Y., Park, P. J., Jung, W. K., & Kim, S. K. (2004). Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein. *European Food Research and Technology, 219*, 20-26.

Jung, W. K., & Kim, S. K. (2007). Calcium-binding peptide derived from pepsinolytic hydrolysates of hoki *(Johnius belengerii)*. *European Food Research and Technology, 224*, 763-767.

Jung, W. K., Je, J. Y., Kim, H. J., & Kim, S. K. (2002). A novel anticoagulant protein from *Scapharca broughtonii*. *Journal of Biochemistry and Molecular Biology, 35*, 199-205.

Jung, W. K., Lee, B. J., & Kim, S. K. (2006). Fish-bone peptide increases calcium solubility and bioavailability in ovariectomised rats. *British Journal of Nutrition, 95*, 124-128.

Jung, W. K., Mendis, E., Je, J. Y., Park, P. J., Son, B. W., Kim, H. C., … Kim, S. K. (2005). Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (*Limanda aspera*) frame protein and its antihypertensive effect in spontaneously hypertensive rats. *Food Chemistry*, *94*, 26-32.

Jung, W. K., Park, P. J., Byun, H. G., Moon, S. H., & Kim, S. K. (2005). Preparation of hoki (*Johnius belengerii*) bone oligophosphopeptide with a high affinity to calcium by carnivorous intestine crude proteinase. *Food Chemistry, 91*, 330-340.

Kagawa, K. F. (2011). *Patent No. PCT/JP2009/068504.* U.S.

Kahn, S. E., Hull, R.L., & Utzschneider, K. M. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature, 444* (14), 840-847.

Kanamoto, R., Azuma, N., Miyamoto, T., Saeki, T., Tsuchihashi, Y., & Iwami, K. (2001). Soybean resistant proteins interrupt an enterohepatic circulation of bile acids and suppress liver tumorigenesis induced by azoxymethane and dietary deoxycholate in rats. *Bioscience Biotechnology and Biochemistry, 4*, 999-1002.

Kastner, M. (2000). *Protein liquid chromatography.* Amsterdam: Elsevier Science.

Kawauchi, H., Moriyama, S., Yasuda, A., Yamaguchi, K., Shirahata, K., Kubota, J., & Hirano, T. (1986). Isolation and characterization of chum salmon growth hormone. *Archives of Biochemistry and Biophysics, 224* (2), 542-552.

Kechaou, E. S., Dumay, J., Moreno, D., Jaouen, P., Gouygou, J. P., Berge, J. P., & Amar, R. B. (2008). Enzymatic hydrolysis of cuttlefish (*Sepia officinalis*) and sardine (*Sardina pilchardus*) viscera using commercial proteases: Effects on lipid distribution and amino acid composition. *Journal of Bioscience and Bioengineering, 107* (2), 158-164.

Khamzina, L., Veilleux, A., Bergeron, S., & Marette, A. (2005). Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. *Insulin-glucagon-gi peptides-diabetes mellitus, 146* (3), 1473-1482.

Khantaphant, S., & Benjakul, S. (2008). Comparative study on the proteases from fish pyloric caeca and the use for production of gelatin hydrolysate with antioxidative activity. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular, 151*, 410-419.

Kim, J. Y., Woo, H. J., Ahn, C. W., Nam, H. S., Shin, Z. I., & Lee, H. J. (1999). Cytotoxic effects of peptides fractionated from bromelain hydrolyzates of soybean protein. *Food Science and Biotechnology, 8*, 333-337.

Kim, S. K., & Mendis, E. (2006). Bioactive compounds from marine processing byproducts – A review. *Journal of Functional Foods, 2*, 1-9.

Kim, S. K., Kim, Y. T., Byun, H. G., Nam, K. S., Joo, D. S., & Shahidi, F. (2001). Isolation and characterization of antioxidative peptides from gelatin hydrolysate of Alaska pollack skin. *Journal of Agricultural and Food Chemistry*, *49*, 1984-1989.

Kim, S. Y., Je, J. Y., & Kim, S. K. (2007). Purification and characterization of antioxidant peptide from hoki (*Johnius belengerii*) frame protein by gastrointestinal digestion. *The Journal of Nutritional Biochemistry, 18* (1), 31-38.

Kim, S., & Wijesekara, I. (2010). Development and biological activities of marine-derived bioactive peptides: A review. *Journal of Functional Foods*, *2*, 1-9.

Kim, S. H., Kim, J. Y., Park, S. W., Lee, K. W., Kim, K. H., & Lee, H. J. (2003). Isolation and purification of anticancer peptides from Korean ginseng. *Food Science and Biotechnology, 12*, 79-82.

Kim, S. K., Ngo, D. H., & Vo, T. S. (2012). Marine fish-derived bioactive peptides as potential antihypertensive agents. *Advances in Food and Nutrition Research, 65*, 249-260.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design, 9*, 1309-1323.

Korhonen, H., & Pihlanto, A. (2005). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*, 945-960.

Kristinsson, H. G. (2008). Functional and bioactive peptides from hydrolyzed aquatic food proteins. In C. &. Barrow (Ed.), *Marine Nutraceuticals and Functional Foods* (pp. 229-246). Boca Raton, FL: CRC Press.

Kristinsson, H. G., & Rasco, B. A. (2000). Fish protein hydrolysates: production, biochemical and functional properties. *Critical Reviews in Food Science and Nutrition, 40* (1), 43-81.

Lavigne, C., Marette, A., & Jacques, H. (2000). Cod and soy proteins compared with casein improve glucose tolerance and insulin sensitivity in rats. *American Journal of Physiology, 278* (3), E491-E500.

Lavigne, C., Tremblay, F., Asselin, G., Jaques, H., & Marette, A. (2001). Prevention of skeletal muscle insulin resistance by dietary cod protein in high fat-fed rats. *American Journal of Physiology: endocrinology and metabolism, 281*, E62-71.

Lazarev, V. N., & Govorun, V. M. (2010). Antimicrobial peptides and their use in medicine. *Applied Biochemistry and Microbiology, 46* (9), 803-814.

Ledesma, B. H., Contreras, M. M., & Recio, I. (2010). Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Advances in Colloid and Interface Science, 165*, 23-35.

Lee, S. H., Qian, Z. J., & Kim, S. K. (2010). A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. *Food Chemistry, 118*, 96-102.

Leung, E. H. W., & Ng, T. B. (2007). A relatively stable antifungal peptide from buckwheat seeds with antiproliferative activity toward cancer cells. *Journal of Peptide Science, 13* (11), 762-767.

Li, Y. (2007). Current progress and advances of study on bioactive peptides. *Food and Fermentation Industries, 33* (1), 1-9.

Li, Y. W., Li, B., He, J. G., & Q, P. (2011). Structure–activity relationship study of antioxidative peptides by QSAR modeling: the amino acid next to C-terminus affects the activity. *Journal of Peptide Science, 17*, 456-462.

Liaset, B., Nortvedt, R., Lied, E., & Espe, M. (2002). Studies on the nitrogen recovery in enzymic hydrolysis of Atlantic salmon (*Salmo salar, L.*) frames by Protamex™ protease. *Process Biochemistry, 37*, 1253-1269.

Lim, C., & Sessa, D. J. (1995). *Nutrition and utilization technology in aquaculture.* Urbana: AOCS Press.

Liu, T. X., & Zhao, M. M. (2010). Thermal pretreatment and chemical modifications as a means to alter hydrolytic characteristics and prevent bitterness in hydrolysates of fishery by-catch (*Decapterus maruadsi*) protein. *International Journal of Food Science & Technology, 45*, 1852-1861.

Lu, J. L., Zeng, Y., Hou, W. R., Zhang, S. S., Li, L. L., Luo, X., …Xiang, M. (2011). The soybean peptide aglycin regulates glucose homeostasis in type 2 diabetic mice via IR/IRS 1 pathway. *Journal of Nutritional Biochemistry.*

Matthews, D. M. (1971). Protein absorption. *Journal of Clinical Pathology, 5,* 29-40.

McGrath, R. (1971). Protein measurement by ninhydrin determination of amino acids released by alkaline hydrolysis. *Analytical Biochemistry, 49*, 95-102.

Meisel, H. (2004). Multifunctional peptides encrypted in milk proteins. *Biofactors, 21*, 55-61.

Mellander, O. (1950). The physiological importance of the casein phosphop-peptide calcium salts. II. Peroral calcium dosage of infants. *Acta Societatis Medicorm Upsaliensis, 55*, 247-255.

Mendis, E., Rajapakse, N., Byun, H. G., & Kim, S. K. (2005). Investigation of jumbo squid (*Dosidicus gigas*) skin gelatin peptides for their in vitro antioxidant effects. *Life Science, 53*, 2166-2178.

Meng, S., Xu, H., & Wang, F. (2010). Research advances of antimicrobial peptides and applications in food industry and agriculture. *Current Protein and Peptide Science, 11* (4), 264-273.

Metzger, B. E. (2006). *American medical association guide to living with diabetes: preventing and treating type 2 diabetes – Essential information you and your family need.* New Jersey: John Wiley & Sons, Inc.

Michael, K. (2000). *Protein liquid chromatography.* Amsterdam: Elsevier Science B. V.

Miller, J. R., Siripurkpong, P., Hawes, J., Majdalawieh, A., Ro, H.-S., & McLeod, R. S., (2008) The trans-10, cis-12 isomer of conjugated linoleic acid decreases adiponectin assembly by PPARγ-dependent and PPARγ-independent mechanisms. *Journal of Lipid Research, 49*, 550-562.

Mine, Y., & Shahidi, F. (2006). *Nutraceutical Proteins and Peptides in Health and Disease.* Florida: Taylor & Francis Group.

Moller, D. (2001). New drug targets for type 2 diabetes and the metabolic syndrome. *Nature, 414* (13), 821-828.

Morimura, S., Nagata, H., Uemura, Y., Fahmi, A., Shigematsu, T., & Kida, K. (2002). Development of an effective process for utilization of collagen from livestock and fish waste. *Process Biochemistry, 37*, 1403-1412.

Moure, A., Dominguez, H., & Parajo, J. C. (2006). Antioxidant properties of ultrafiltration recovered soy protein fractions from industrial effluents and their hydrolysates. *Process Biochemistry, 41*, 447-456.

Mozzi, F., Raya, R. R., & Vignolo, G. M. (2010). *Biotechnology of Lactic acid bacteria: novel applications*. Amsterdam: Blackwell publishing.

Nagodawithana TW, Nelles L, & Trivedi NB. (2010). *Protein hydrolysates in biotechnology.* The Netherlands: Springer.

Nagodawithana, T. W. (1998). *Nutritional requirements of commercially important microorganisms*. Milwaukee: Esteekay Associates.

Nakaoka, F., Sasakawa, Y., Yamamoto, K., Nakao, M., Nakamura, M., Tong, C., Fukuhama, C., & Kagawa, K. (2010). Anti-diabetic effects of globin digest and its active ingredient Leu-Ser-Glu-Leu in ICR mice, streptozotocin-induced diabetic mice and KK-Ay mice. *Life Science, 86* (11-12), 424-434.

Nielsen, S. S. (2010). *Food analysis.* New York: Springer.

Nigam, A., & Ayyagari, A. (2007). *Lab manual in biochemistry, immunology and biotechnology.* New Delhi: Tata McGraw-Hill Publishing Company.

Ohba, R., Deguchi, T., Kishikawa, M., Arsyad, F., Morimura, S., & Kida, K. (2003). Physiological functions of enzymatic hydrolysates of collagen or keratin contained in livestock and fish waste. *Food Science and Technology Research, 9*, 91-93.

Orozco, L. J., Buchleitner, A. M., Gimenez, P. G., Roquel, F. M., Richter, B., & Mauricio, D. (2008). Exercise or exercise and diet for preventing type 2 diabetes mellitus. *Cochrane Database Systematic Reviews, 16* (3), CD003054.

Ouellet, V., Marois, J., Weisnagel, S. J., & Jacques, H. (2007). Dietary cod protein improves insulin sensitivity in Insulin-resistant men and women. *Diabetes Care*, *30*, 2816-2821.

Papagianni, M. (2003). Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. *Biotechnology Advances, 21* (6), 465-499.

Pasupuleti V. K., & Braun S. (2010). *Protein hydrolysates in biotechnology.* New York: Springer.

Peacock, J. (2000). *Diabetes.* Mankato: Capstone Press.

Pedroche, J., Yust, M. M., Lqari, H., Girón-Calle, J., Vioque, J., Alaiz, M., & Millan, F. (2004). Production and characterization of casein hydrolysates with a high amino acid Fisher's ratio using immobilized proteases. *International Dairy Journal, 14*, 527-533.

Petsko, G. A., & Ringe, D. (2004). *Protein structure and function.* London: New Science Press Ltd.

Phanturat, P., Benjakul, S., Visessanguan, W., & Roytrakul, S. (2010). Use of pyloric caeca extract from bigeye snapper (*Priacanthus macracanthus*) for the production of gelatin hydrolysate with antioxidative activity. *LWT-Food Science and Technology, 43*, 86-97.

Picot, L., Bordenave, S., Didelot, S., Fruitier-Arnaudin, I., Sannier, F., Thorkelsson, G., … Piot, J. M. (2006). Antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines. *Process Biochemistry, 41*, 1217-1222.

Pilon, G., Dallaire, P., & Marette, A. (2004). Inhibition of inducible nitric-oxide synthase by activators of AMP-activated protein kinase - A new mechanism of action of insulin-sensitizing drugs. *Journal of Biological Chemistry*, *279* (20), 20767-20774.

Pilon, G., Ruzzin, J., Rioux, L. E., Lavinge, C., Frøyland, L., Jacques, H., …Marette, A. (2010). Differential effects of various fish proteins in altering body weight, adiposity, inflammatory status, and insulin sensitivity in high-fat-fed rats. *Metabolism, 12*, 005.

Potter, R., Hansen, L. T., & Gill, T. A. (2005). Inhibition of foodborne bacteria by native and modified protamine: Importance of electrostatic interactions. *International Journal of Food Microbiology, 103* (1), 23-34.

Pupovac, J., & Anderson, G. H. (2002). Dietary peptides induce satiety via cholecystokinin-A and peripheral opioid receptors in rats. *Journal of Nutrition, 13*, 2775-2780.

Qian, Z. J., Je, J. Y., & Kim, S. K. (2007). Antihypertensive effect of angiotensin I converting enzyme-inhibitory peptide from hydrolysates of bigeye tuna dark muscle, *Thunnus obesus*. *Journal of Agricultural and Food Chemistry, 55*, 8398–8403.

Qian, Z. J., Jung, W. K., & Kim, S. K. (2008). Free radical scavenging activity of novel antioxidative peptide purified from hydrolysate of bullfrog skin, *Rana catesbeiana Shaw*. *Bioresource Technology, 99*, 1690-1698.

Rajapakse, N., Jung, W. K., Mendis, E., Moon, S. H., & Kim, S. K. (2005). A novel anticoagulant purified from fish protein hydrolysate inhibits factor XIIa and platelet aggregation. *Life Science*, *76*, 2607-2619.

Rajapakse, N., Mendis, E., Byun, H. G., & Kim, S. K. (2005). Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *Journal of Nutritional Biochemistry, 16*, 562-569.

Ravichandran, S., Kumaravel, K., Rameshkumar, G., & Ajithkumar, T. T. (2010). Antimicrobial peptides from the marine fishes. *Research Journal of Immunology, 3*(2), 146-156.

Reaven, G. M. (1988). Role of insulin resistance in human disease. *Diabetes*, 1595-1607.

Reddy, K. V. R., Yedery, R. D., & Aranha, C. (2004). Antimicrobial peptides: premises and promises. *International Journal of Antimicrobial Agents, 24*, 536-547.

Reed, R. H., Holmes, D., Weyers, J., & Jones, A. (2007). *Practical skills in biomolecular sciences* (3rd ed). London: Pearson Education Ltd.

Rosenthal, M. S. (1999). *The type 2 diabetic woman.* Illinois: Lowell House.

Santos, S. D., Martins, V. G., Mellado, M., & Prentice, C. (2011). Evaluation of functional properties in protein hydrolysates from bluewing searobin (*Prionotus punctatus*) obtained with different microbial enzymes. *Food Bioprocess technology, 4*, 1399-1406.

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides, 31* (10), 1949-1956.

Sathivel, S., Bechtel, P. J., Babbitt, J., Smiley, S., Crapo, C., Reppond, K. D., & Prinyawiwatkul, W. (2003). Biochemical and functional properties of herring (*Clupea harengus*) byproduct hydrolysates. *Journal of Food Science, 68*, 2196-2200.

Schlamowitz, M., Varandani, P. T., & Wissler, F. C. (1963). Pepsinogen and Pepsin: Conformational Relations, Studied by Iodination, Immunochemical Precipitation, and the Influence of Pepsin Inhibitor. *Biochemistry*, *2* (2), 238-246.

Serrano Rios, M., & Gutierrez Fuentes, J. A. (2009). *Type 2 diabetes mellitus*. Barcelona: Elsevier Espana, S.L.

Sewald, N., & Jakubke, H. D. (2009). *Peptides: Chemistry and Biology.* Germany: Wiley.

Shahidi, F., & Zhong, Y. (2008). Bioactive peptides. *Journal of AOAC international, 91* (4), 914-933.

Sheehan, D. (2009). *Biochemistry: Principle and Applications.* UK: John Wiley& Sons Ltd.

Sheih, I. C., Fang, T. J., Wu, T. K., & Lin, P. H. (2010). Anticancer and Antioxidant Activities of the Peptide Fraction from algae protein waste. *Journal of Agricultural and Food Chemistry, 58*, 1202-1207.

Shi, X. D., & Chen, X. L. (2000). Process in studies of marine bioactive peptides. *Amino Acids & Biotic Resources, 22* (4), 13-16.

Shimadzu. Gel Permeation Chromatography System - Application Data Book. C190-0032, p. 81

Sivasankar, B. (2005). *Bioseparations: principles and techniques.* New Delhi: Prentice-Hall of India Private Limited.

Slizyte, R. Dauksas, E., Falch, E., Storr, I., & Rustad, T. (2005). Characteristics of protein fractions generated from hydrolysed cod (*Gadus morhua*) by-products. *Process Biochemistry, 40* (6), 2021-2023.

Snyder, L. R., Kirkland, J. J., & Glajch, J. L. (1997). *Practical HPLC method development (2nd ed).* New York: John Wiley & Son.

Stephanie, S. (2009). *Healthy eating for type 2 diabetes.* Boston: Harvard Medical School.

Sun, J., He, H., & Xie, B. J. (2004). Novel antioxidant peptides from fermented mushroom Ganoderma lucidum. *Journal of Agricultural and Food Chemistry, 52*, 6646-6652.

Taskaya, L., Chen, Y. C., & Jaczynski, J. (2009). Functional properties of proteins recovered from silver recovered from silver carp (*Hypophthalmichthys molitrix*) by isoelectric solubilization / precipitation. *LWT – Food Science and Technology, 42* (6), 1082-1089.

Thermo Fisher Scientific, 2008. Peptide solubility. *TI-PEP08-0708.*

Thomas, D., & Loffler, F. (1994). Improved protein functionalities by enzymatic treatment. *Food Marketing & Technology, 2*, 4-6.

Thorkelsson, G., & Kristinsson, H. G. (2009). *Bioactive peptides from marine sources.* Reykjavik: State of art.

Tremblay, F., Lavigne, C., Jaques, H., & Marette, A. (2003). Dietary cod protein restores insulin-induced activation of phosphatidylinositol 3-kinase Akt and GLUT4 translocation to the T-tubules in skeletal muscle of high-fat-fed obese rats. *Diabetes, 52*, 29-37.

Udenigwe, C. C., & Aluko, R. E. (2011). Food Protein-Derived Bioactive Peptides: Production, Processing, and Potential Health Benefits. *Journal of Food Science*, *71,* R11-R24.

Unwin, N., Gan, D., & Whiting, D. (2010). The IDF Diabetes Atlas: providing evidence, raising awareness and promoting action. *Diabetes Research and Clinical Practice, 87*, 2-3.

Van Regenmortel, M. H. V., & Mulller, S. (1999). *Synthetic peptides as antigens.* Amsterdam: Elsevier Science B. V.

Vermeirssen V., Van Camp, J., & Verstraete, W. (2004). Bioavailability of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition, 92*, 357-366.

Virally, M., Blickle, J. F., Girard, J., Halimi, S., Simon, D., & Gulliausseau, P. J. (2007). Type 2 diabetes mellitus: epidemiology, pathophysiology, unmet needs and therapeutical perspectives. *Diabetes and Metabolism, 33* (4), 231-244.

Webster, J. D., Ledward, D. A., & Lawrie, R. A. (1982). Protein hydrolysates from meat industry by-products. *Meat science*, *7* (2), 147-157.

Winsdor, M. L. (2001). Fish meal. *Torry Advisor Notes , 49*.

Wu, J., & Ding, X. (2002). Characterization of inhibition and stability of soy-protein-derived angiotensin I-converting enzyme inhibitory peptides. *Food Research International, 35* (4), 367.

Yagasaki, M. (2009). Fermentation technology breakthrough for the formation of dipeptides. *Nutracon.* Anaheim.

Yang, R., Zhang, Z., Pei, X., Han, X., Wang, J., Wang, L., … Li, Y. (2009). Immunomodulatory effects of marine oligopeptide preparation from Chum Salmon (*Oncorhynchus keta*) in mice. *Food Chemistry, 113*, 464-470.

Yi, H. J., Kim, J. Y., Kim, K. H., Lee, H. J., & Lee. H. J. (2003). Anticancer activity of peptide fractions from egg white hydrolysate against mouse lymphoma cells. *Food Science and Biotechnology, 12*, 224-227.

Yuan, X. Q., Gu, X. H., & Tang, J. (2008). Purification and characterisation of a hypoglycemic peptide from *Momordica Charantia L. Var. abbreviata Ser*. *Food Chemistry, 111* (2), 415-420.

Zachariou, M. (2008). *Affinity Chromatography: Methods and Protocols.* New Jersey: Humana Press.