การผลิตและการศึกษาลักษณะเพปไทด์ที่มีคุณสมบัติในการต้านออกซิเดชันจากวัสดุเหลือทิ้งจากซูริมิโดยใช้เอนไซม์

นางสาวชมภูนุช วิริยะพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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PRODUCTION AND CHARACTERIZATION OF ANTIOXIDANT PEPTIDES DERIVED FROM ENZYMATIC HYDROLYSIS OF SURIMI WASTES

Chompoonuch Wiriyaphan

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

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Academic Year 2013

PRODUCTION AND CHARACTERIZATION OF ANTIOXIDANT PEPTIDES DERIVED FROM ENZYMATIC HYDROLYSIS OF SURIMI WASTES

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ชมภูนุช วิริยะพันธ์ : การผลิตและการศึกษาลักษณะเพปไทด์ที่มีคุณสมบัติในการต้านออกซิเดชันจากวัสดุเหลือทิ้งจากซูริมิโดยใช้เอนไซม์ (PRODUCTION AND CHARACTERIZATION OF ANTIOXIDANT PEPTIDES DERIVED FROM ENZYMATIC HYDROLYSIS OF SURIMI WASTES) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 205 หน้า.

ในกระบวนการผลิตซูริมิมีวัสดุเหลือทิ้งที่เป็นของแข็งจำนวนมาก การผลิตโปรตีนไฮโดรไลเสทที่มีคุณสมบัติเป็นสารต้านอนุมูลอิสระเป็นแนวทางหนึ่งในการใช้ประโยชน์และเพิ่มมูลค่าของวัสดุเหลือทิ้งเหล่านี้ วัตถุประสงค์ของการศึกษานี้คือเพื่อผลิตโปรตีนไฮโดรไลเสทที่มีคุณสมบัติเป็นสารต้านอนุมูลอิสระจากวัสดุเหลือทิ้งของกระบวนการผลิตซูริมิจากปลาทรายแดง รวมถึงก้าง กระดูก และ เศษหนัง และวัสดุเหลือทิ้งจากกระบวนการกำจัดเนื้อเยื่อเกี่ยวพัน โดยใช้โปรติเนสแหล่งใหม่ที่ผลิตจากแบคทีเรียสายพันธุ์ *Virgibacillus* sp. SK33 และโปรติเนสทางการค้า คือ เพปซิน ทริปซิน และ อัลคาเลส โปรตีนไฮโดรไลเสทจากก้าง กระดูกและเศษหนังที่ผ่านการย่อยโดยเพปซินมีฤทธิ์ต้านอนุมูลอิสระสูงที่สุด จากการทดสอบด้วยอนุมูลอิสระ 2, 2'-azinobis (3-ethyl-benzothiazoline-6-sulfonate (ABTS) ความสามารถในการรีดิวซ์เหล็กเฟอริค และความสามารถในการยับยั้งการฟอกสีของเบต้าแคโรทีน โปรตีนไฮโดรไลเสทจากก้าง กระดูก และเศษหนังมีฤทธิ์ในการต้านอนุมูลอิสระสูงกว่าโปรตีนไฮโดรไลเสทจากของเหลือทิ้งจากกระบวนการกำจัดเนื้อเยื่อเกี่ยวพัน ($p<0.05$) อย่างไรก็ตาม โปรตีนไฮโดรไลเสทจากวัตถุดิบทั้งสองแหล่ง สามารถป้องกันเซลล์ HepG2 จากออกซิเดชันที่เหนี่ยวนำโดยเทอร์-บิวทิลไฮโดรเพอร์ออกไซด์ (*tert*-butyl hydroperoxide: TBHP) ไม่แตกต่างกัน ($p>0.05$)

แยกโปรตีนไฮโดรไลเสทจากก้าง กระดูก และเศษหนังที่ผ่านการย่อยโดยโปรติเนส จากแบคทีเรียสายพันธุ์ *Virgibacillus* sp. SK33 ด้วยเทคนิคโครมาโทกราฟีแบบแลกเปลี่ยนประจุลบ (anion exchange chromatography) และแยกตามขนาด (size exclusion chromatography) หลังจากแยกด้วยเทคนิคโครมาโทกราฟีตามขนาดได้เพปไทด์ 3 ส่วน คือ B1 B2 และ B3 โดยเพปไทด์ B3 มีความสามารถในการจับกับอนุมูล ABTS และมีสมบัติรีดิวซ์เหล็กสูงสุด ในขณะที่ส่วน B2 และ B3 มีความสามารถเด่นในการจับกับเหล็กและอนุมูลไฮดรอกซิล นอกจากนี้ ส่วนเพปไทด์ในส่วน B1 และ เพปไทด์สังเคราะห์ (FLGSFLYEYSR) ที่ได้มาจากการวิเคราะห์ลำดับเพปไทด์จากส่วน B3 มีความสามารถในการจับอนุมูลอิสระที่เกิดจากไฮโดรเจนเปอร์ออกไซด์ภายในเซลล์ HepG2

แยกโปรตีนไฮโดรไลเสทจากก้าง กระดูก และเศษหนังที่ผ่านการย่อยโดยโปรติเนสจากแบคทีเรียสายพันธุ์ *Virgibacillus* sp. SK33 ด้วยเทคนิคอัลตราฟิลเตชันได้ 4 ส่วน คือ FBSH-I (>30

kDa) FBSH-II (5-30 kDa) FBSH-III (1-5 kDa) และ FBSH-IV (<1 kDa) FBSH-III มีฤทธิ์ในการเป็นสารต้านอนุมูลอิสระสูงสุดโดยตรวจสอบด้วยอนุมูลอิสระทางเคมีและชีวภาพ ความเข้มข้นของเพปไทด์ FBSH-III ในช่วง 25 ถึง 200 ไมโครกรัมต่อมิลลิลิตร สามารถป้องกันเซลล์ตายจากการเหนี่ยวนำความเป็นพิษโดย TBHP และสามารถยับยั้งการซึมออกของเอนไซม์ แลคเตต ดีไฮโดรจีเนส (Lactate dehydrogenase: LDH) และยับยั้งการผลิตอนุมูลอิสระในเซลล์ Caco-2 นอกจากนี้ FBSH-III ยังคงแสดงฤทธิ์ต้านอนุมูลหลังจากผ่านการให้ความร้อน (40-121 องศาเซลเซียส) ความเป็นกรดด่าง (pH 2.0-10.0) และหลังการย่อยโดยเอนไซม์ในระบบทางเดินอาหารแบบจำลอง ผลการศึกษานี้แสดงให้เห็นว่าก้าง กระดูก และเศษหนังมีศักยภาพเป็นแหล่งโปรตีนสำหรับผลิตเพปไทด์ที่มีคุณสมบัติเป็นสารต้านอนุมูลอิสระ และอาจใช้เป็นผลิตภัณฑ์โภชนเภสัชได้

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา_____

CHOMPOONUCH WIRIYAPHAN : PRODUCTION AND CHARACTERIZATION OF ANTIOXIDANT PEPTIDES DERIVED FROM ENZYMATIC HYDROLYSIS OF SURIMI WASTES. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWADIGUL, Ph.D., 205 PP.

SURIMI WASTES/THREADFIN BREAM/*VIRGIBACILLUS* SP. SK33/PEPTIDES/ PROTEIN HYDROLYSATE/ANTIOXIDANT ACTIVITY/CYTOPROTECTIVE EFFECT

A large amount of solid waste is generated in surimi production. Production of protein hydrolysate with antioxidant properties is one approach to fully utilize and increase the value of this waste. The objectives of this study were to produce protein hydrolysates with antioxidant properties from threadfin bream surimi waste, including frame, bone and skin (FBS) and refiner discharge (RD) using proteinases from a novel source, *Virgibacillus*sp. SK33, and commercial proteinases: pepsin, trypsin and Alcalase. Pepsin-hydrolyzed FBS showed the highest antioxidant activity based on 2, 2'-azinobis (3-ethyl-benzothiazoline-6-sulfonate (ABTS) radical scavenging, ferric reducing antioxidant power (FRAP) and inhibition of β-carotene bleaching assays. FBS hydrolysates showed higher antioxidant activity based on chemical assays than their RD counterparts. However, FBS and RD hydrolysates protected HepG2 cells against *tert*-butyl hydroperoxide (TBHP)-induced oxidative damage to a similar extent.

FBS protein hydrolysates prepared from *Virgibacillus* sp. SK33 proteinase was further fractionated using anion exchange and size exclusion chromatography

(SEC). Three fractions, namely, B1, B2 and B3, were obtained after SEC. Fraction B3 exhibited the highest ABTS scavenging activity and FRAP value, while metal chelation and hydroxyl radical scavenging ability were distinctive in fractions B2 and B3. Fraction B1 and a synthetic peptide selected from the pooled *de novo* peptides of fraction B3, FLGSFLYEYSR, had a cellular radical scavenging effect when HepG2 cells were treated with hydrogen peroxide (H_2O_2).

FBS hydrolysate prepared from *Virgibacillus* sp. SK33 proteinase was fractionated using ultrafiltration membranes into four fractions, namely FBSH-I (>30 kDa), FBSH-II (5-30 kDa), FBSH-III (1-5 kDa), and FBSH-IV (<1 kDa). FBSH-III showed the highest antioxidant activity based on chemical and biological assays. At 25-200 µg/ml, FBSH-III showed a protective effect against TBHP-induced cytotoxicity Caco-2 cells. It also inhibited lactate dehydrogenase (LDH) leakage and intracellular reactive species (ROS) production in Caco-2 cells. Furthermore, this fraction retained antioxidant activity after various thermal (40-121 °C) and pH treatments (pH 2.0-10.0) as well as *in vitro* simulated gastrointestinal (GI) digestion. This study suggests that FBS could be a potential source for antioxidant peptides production and therefore could be developed as a promising nutraceutical product.

School of Food Technology Student's Signature _____

Academic Year 2013 Advisor's Signature _____

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LIST OF ABBREVIATIONS

ABTS	=	2, 2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AH	=	Hydrolysate prepared from Alcalase
Ala (A)	=	Alanine
ANS	=	8-anilino-1-naphthalenesulfonic acid
Arg (R)	=	Arginine
Asn (N)	=	Asparagine
Asp (D)	=	Aspartic acid
°C	=	Degree celsius
Ca	=	Calcium
CAT	=	Catalase
Cys (C)	=	Cysteine
AMC	=	Aminomethylcoumarin
AOAC	=	Association of Official Chemists
BSA	=	Bovine serum albumin
Caco-2	=	Human adenocarcinoma colon cancer
DCF	=	2’, 7’-dichlorofluorescin
DCFH-DA	=	2’, 7’-dichlorofluorescin diacetate
DEAE	=	Diethylaminoethyl
DH	=	Degree of hydrolysis
DPPH	=	2, 2-diphenyl-1-picrylhydrazyl
DMEM	=	Dulbecco’s modified eagle’s medium

LIST OF ABBREVIATIONS (Continued)

DMEM	=	Dulbecco's modified eagle's medium
DMEM-F12	=	Dulbecco's ModifiedEagle Media: Nutrient Mixture F-12
DMSO	=	Dimethyl sulfoxide
EDTA	=	Ethylenediaminetetraacetic acid
E_m	=	Emission wavelength
ESI	=	Electrospray ionization
ET	=	Electron transfer
E_x	=	Excitation wavelength
FBS	=	The mixture of frame, bone and skin
FBSH	=	FBS hydolysates
FBSH-I	=	FBS hydrolysate was not permeated the 30 kDa membrane
FBSH-II	=	FBS hydrolysate was permeated the 30 kDa membrane but not permeated the 5 kDa membrane
FBSH-III	=	FBS hydrolysate was permeated the 5 kDa membrane but not permeated the 1 kDa membrane
FBSH-IV	=	FBS hydrolysate was permeated the 1kDa membrane
Fe	=	Iron
FRAP	=	Ferric reducing antioxidant power
GI	=	Gastrointestinal
Glu (E)	=	Glutamic acid
Gln (Q)	=	Glutamine
Gly (G)	=	Glycine

LIST OF ABBREVIATIONS (Continued)

GPx	=	Glutathione peroxidase
H	=	Hour
His (H)	=	Histidine
H_0	=	Surface hydrophobicity
HAT	=	Hydrogen atom transfer
HBSS	=	Hank's balanced salt solution
HepG2	=	Human hepatomacarcinoma
H_2O_2	=	Hydrogen peroxide
$^{\bullet}OH$	=	Hydroxyl radical
Hyp (O)	=	Hydroxyproline
IC_{50}	=	Inhibitory concentration 50 (concentration of inhibitory agent required to inhibit the activity by 50%)
ICP-OES	=	Inductive Coupled Plasma-Optical Emission Spectroscopy
Ile (I)	=	Isoleucine
K	=	Potassium
kDa	=	kilo Dalton (10^3 Dalton)
LC-MS/MS	=	Liquid chromatography with tandem mass spectrometry
Leu (L)	=	Leucine
Lys (K)	=	Lysine
LOOH	=	Lipid peroxide
M	=	mol l^{-1}
Met (M)	=	Methionine

LIST OF ABBREVIATIONS (Continued)

Mg	=	Magnesium
µg	=	Microgram (10^{-6} gram)
µl	=	Microliter (10^{-6}l)
µM	=	Micromolar (10^{-6}mol l^{-1})
mg	=	Milligram (10^{-3}gram)
min	=	Minute
ml	=	Milliliter (10^{-3}l)
mM	=	Millimolar (10^{-3}mol l^{-1})
MS	=	Mass spectrometry
MS/MS	=	Tandem mass
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Mw	=	Molecular weight
MWCO	=	Molecular weight cut off
m/z	=	Mass per charge ratio
Na	=	Sodium
Nm	=	Nanometer (10^{-9} meter)
$O_2^{-\bullet}$	=	Superoxide radical
$^1O_2$	=	Singlet oxygen
OD	=	Optical density
ORAC	=	Oxygen radical absorbance capacity
P	=	Phosphorus
PAGE	=	Polyacrylamide gel electrophoresis

LIST OF ABBREVIATIONS (Continued)

PBS	=	Phosphate buffer saline
PH	=	Hydrolysate prepared from pepsin
Phe (F)	=	Phenylalanine
Pro (P)	=	Proline
RD	=	Refiner discharge
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
$RO^{\bullet}$	=	Alkoxyl radical
$ROO^{\bullet}$	=	Peroxyl radical
ROOH	=	Hydroperoxide
RP-HPLC	=	Reversed-phase high performance liquid chromatography
S	=	Second
SDS	=	Sodium dodecyl sulfate
SEC	=	Size exclusion chromatography
Ser (S)	=	Serine
SOD	=	Superoxide dismutase
TBA	=	Thiobarbituric acid
TBHP	=	*Tert*-butyl hydroperoxide
TCA	=	Trichloroacetic acid
TFA	=	Trifluoroacetic acid
TH	=	Hydrolysate prepared fromtrypsin
Thr (T)	=	Threonine

LIST OF ABBREVIATIONS (Continued)

TNBS	=	2, 4, 6-trinitrobenzenesulfonic acid
TPTZ	=	2, 4, 6-tripyridyl-s-triazine
Tris	=	Tris (hydroxymethyl) aminomethane
Trp (W)	=	Tryptophan
TTHP	=	Total hydrophobic amino acid
Tyr (Y)	=	Tyrosine
U	=	Unit activity
UF	=	Ultrafiltration
VH	=	Hydrolysate prepared from *Virgibacillus* sp. SK 33
Val (V)	=	Valine
w/v	=	Weight by volume
xg	=	Gravitational acceleration

CHAPTER I

INTRODUCTION

1.1 Introduction

Reactive oxygen species (ROS) are molecules, elements, atoms or radicals composing of oxygen in the structure and highly reactive. In aerobic organisms, ROS are produced during normal physiological processes, such as energy production, metabolism or generation of defenses against invasive microorganisms. ROS are superoxide radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), alkoxy radical ($RO^{\bullet}$), singlet oxygen ($^1O_2$) and hydroxyl radical ($HO^{\bullet}$), including lipid peroxide (LOOH) or hydroperoxide (ROOH) (Cornelli, 2009). These radicals are very unstable and react rapidly with other groups or substances in the body, leading to cell or tissue injury. In addition, metal ions are known to induce free radical formation through a variety of processes, including the Fenton reaction (Halliwell, Aeschbach, Loliger, and Aruoma, 1995). Under physiological conditions, cells are protected against autoxidation by antioxidants, both enzymatic and non-enzymatic systems. The enzymatic systems include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). For non-enzymatic systems, the small-molecule antioxidants, such as vitamin C, vitamin E, glutathione and ubiquinol act as hydrogen and/or electron donors and transferrin, ferritin, lactoferrin and albumin act as metal chelators (Chow, 1988). Oxidative stress occurs when ROS overload the body's antioxidant defenses or when the antioxidant defense system loses its capacity for response. The excessive ROS can

damage DNA, proteins, lipids, leading to many serious diseases like cancers, inflammatory and the liver diseases (Loguercio and Federico, 2003; Simmonds and Rampton, 1993; Vitaglione, Morisco, Caporaso, and Fogliano, 2004). Besides physiological effect, free radicals affect both the rate of autoxidation, causing rancidity and toxicity (Nawar, 1996). Synthetic antioxidants, such as 3-ter-butyl-4-hydroxyanisole (BHA), 3, 5-di-tert-butyl-4-hydroxytoluene (BHT), tertiary-butylhydroxyquinone (TBHQ) and propyl gallate, are commonly used in food industry to retard lipid oxidation. However, BHA and BHT are likely to cause liver damage and carcinogenesis (Grice, 1986; Wichi, 1988). It is, therefore, of great interest both from academic and practical points of view in exploring alternative antioxidants from many natural sources that enhance antioxidative defenses in the body and retard lipid oxidation in food systems.

Some food proteins, particularly protein hydrolysate prepared by enzymatic hydrolysis, have been reported to exhibit antioxidant activity, such as soybean (Chen, Muramoto, Yamauchi, and Nokihara, 1996), porcine myofibrillar (Saiga, Tanabe, and Nishimura, 2003), porcine collagen (Li, Chen, Wang, Ji, and Wu, 2007), tuna backbone (Je, Qian, Byun, and Kim, 2007), hoki frame (Kim, Je, and Kim, 2007), potato (Kudo, Onodera, Takeda, Benkeblia, and Shiomi, 2009), royal jelly from the honeybee (Guo, Kouzuma, and Yonekura, 2009), Nile tilapia scale (Ngo, Qian, Ryu, Park, and Kim, 2010), barley glutelin (Bamdad, Wu, and Chen, 2011), barley hordein (Xia, Bamdad, Ganzle, and Chen, 2012), abalone viscera (Zhou et al., 2012), rapeseed (He, Girgih, Malomo, Ju, and Aluko, 2013), and tilapia (Sun, Zhang, and Zhuang, 2013). Antioxidant activities of those protein hydrolysates are based on protein substrate, hydrophobicity, degree of hydrolysis, molecular mass of peptides, amino

acid compositions and sequences. Hydrolysates rich in peptides containing hydrophobic amino acids, such as Pro, Leu, Ala, Trp and Phe, are believed to possess high antioxidant activity (Mendis, Rajapakse, and Kim, 2005). Peptides containing Trp, Tyr and Met also show the highest antioxidant activity, followed by Cys, His and Phe (Dávalos, Miguel, Bartolomé, and López-Fandiño, 2004). In addition, acidic (Asp and Glu) and/or basic amino acids (Lys, His, and Arg) play an important role in the chelation of metal ions by carboxyl and amino groups in their side chains (Rajapakse, Mendis, Byun, and Kim, 2005; Zhang et al., 2010). Amino acid composition of peptides in protein hydrolysate is entirely dependent on the protein substrates and proteinases used.

Enzymatic hydrolysis is widely applied to improve and upgrade the functional and nutritional properties of food proteins (Zhu, Zhou, and Qian, 2006). Commercial proteinases, such as Alcalase, neutrase, papain, pepsin, α-chymotrypsin and trypsin can be used to hydrolyze bullfrog skin protein for the production of protein hydrolysates with antioxidant property and Alcalase was the most effective of the six proteinases used (Qian, Jung, and Kim, 2008). In addition, trypsin and pepsin were reported to improve antioxidant activity of giant squid muscle and bigeye tuna dark muscle byproduct hydrolysate, respectively (Je, Qian, Lee, Byun, and Kim, 2008; Rajapakse et al., 2005). However, the use of proteinases from novel sources in protein hydrolysate production has not been widely studied. *Virgibacillus* sp. SK33 isolated from one-month-old Thai fish sauce mashes is a potential source of extracellular proteinases with broad specificity toward oxidized insulin B with the dominant cleavage sites at Tyr^{16}-Leu^{17} and Phe^{25}-Tyr^{26} (Sinsuwan, Rodtong, and Yongsawatdigul, 2010). Peptides containing amino acid residues exhibiting

antioxidant activity would be obtained through hydrolytic reaction of *Virgibacillus* sp. SK33 proteinases. This could be a potential processing-aid for production of bioactive peptides with antioxidant activity.

Thailand is one of the largest surimi producers in Southeast Asia (Guenneugues and Morrissey, 2005). The production of surimi increased from 75,000 MT in 2009 to about 94,000 MT in 2011 (http://www.groundfishforum.com). Threadfin bream is one of the most important raw materials (Morrissey and Tan, 2000). The quantity of threadfin bream raw materials supplied to surimi production is estimated to be 200,000 MT/year (Pangsorn, Laong-manee, and Siriraksophon, 2007). Normally, the solid wastes include frame and skin/bone (FBS) and refiner discharges (RD) from deboning machines and a refining process are about 22-27 and 5%, respectively (Morrisey, Lin, and Ismond, 2005). It is, therefore, estimated that FBS, as well as RD from threadfin bream alone are estimated to be 50,000-60,000 MT annually. These wastes are usually converted to low value fish meal. A means to fully utilize and increase marketability of these wastes should be sought.

Amino acid sequencing and characterization of individual peptides could be important for studying mechanism and may provide information about the characteristics of bioactive peptide (Raghavan, Kristinsson, and Leeuwenburgh, 2008). In addition, amino acid sequences will be useful for the peptide synthesis for either food or pharmaceutical industry. However, a critical step toward commercializing fish protein hydrolysates as a nutraceutical product would involve evaluation of antioxidant properties of the hydrolysate as a whole, rather than identifying individual peptide (Raghavan et al., 2008). In addition, protein hydrolysate can have certain benefits over those of purified peptides since the absorption of

oligopeptides can be increased in the presence of sugar and amino acids (Pappenheimer and Volpp, 1992). An industrial process, the antioxidant peptides need to be produced in a continuous bulk process. Ultrafiltration (UF) is a simple and economic process that has been successfully used to concentrate antioxidant peptides from soy protein (Moure, Domínguez, and Parajó, 2006). In addition, the molecular weight distribution of the desired hydrolysates can be controlled through a selection of UF membrane (Je et al., 2007).

Stability of antioxidant peptides during food processing should be assessed before commercializing a particular protein hydrolysate of interest for incorporation into a food product (Samaranayaka and Li-Chan, 2011). Nalinanon, Benjakul, Kishimura, and Shahidi (2011) reported that a small loss in antioxidant activity of peptides might be due to either degradation or aggregation of some antioxidant peptides, caused by heat treatment. In addition, antioxidant peptides might undergo the conformation changes at high alkaline and acidic pH, leading to the loss in their ability (Klompong, Benjakul, Kantachote, Hayes, and Shahidi, 2008). Furthermore, when peptides pass through the gastrointestinal (GI) tract, they could be modified through GI proteinases, which could consequently alter antioxidant properties (Chen and Li, 2012). Although animal studies and human clinical trials are the best ways to study the bioactivity and bioavailability of functional ingredients, *in vitro* simulated GI digestion is being extensively used. It allows for rapid and inexpensive study of bioactive compounds for assessment of their efficacy in *vivo* (Cinq-Mars, Hu, Kitts, and Li-Chan, 2008). Therefore, effects of thermal, pH, and *in vitro* GI tract model on stability of antioxidant peptides should be addressed.

Due to a wide variety of oxidation processes and antioxidative action of protein

hydrolysates or peptides, the use of a single method to evaluate the antioxidant activity cannot provide a clear idea about its real antioxidant potential (Ktari et al., 2012). Peptides with pronounced antioxidant activity *in vitro* based on synthetic radical scavenging assays may exert little or no activity *in vivo*. Most of these assays including 2, 2'-azinobis (3-ethyl-benzothiazoline-6-sulfonate (ABTS), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP) assays are conducted under non-physiological condition. For this reason, the results might not necessarily correlate with biological responses. However, radical scavenging assays are simple, inexpensive, rapid and helpful in evaluating the antioxidant mechanisms of a compound. A step closer to *in vivo* is the use of the *in vitro* cellular models. Determination of antioxidant activity in cell culture model represents a more biologically relevant method than chemical assays of antioxidant activity because it accounts for some aspects of the uptake, metabolism, and location of antioxidants within cells (Carrasco-Castilla et al., 2012).

The mechanisms of cytoprotection relevant to the small intestinal epithelium or liver using the *in vitro* cellular models are of particular interest (Lima, Andrade, Seabra, Fernandes-Ferreira, and Pereira-Wilson, 2007; Schantz, Mohn, Baum, and Richling, 2010). Gastrointestinal (GI) tract is known to be a main site of oxidation in human body since it is exposed to food derived Fe, Cu, H_2O_2, NO, heme, lipid peroxides, and aldehydes, and is an important site of various diseases (Crohn's disease, ulcerative colitis or colon and rectum cancer) (Halliwell, Zhao, and Whiteman, 2000; Simmonds and Rampton, 1993). Liver is also high metabolic activity organ and its anatomical position permits the receiving of blood directly from the gastrointestinal tract. It is vulnerable to toxicity from a variety of drugs and

environmental contaminants.

HepG2 cell line derived from a human hepatocarcinoma, can display highly morphological and functional differentiation resembling normal human hepatocytes under proper culture (Narayanan, Fitch, and Levenson, 2001). Caco-2 cells, derived from a human intestinal adenocarcinoma, exhibit spontaneous enterocyte-like differentiation, showing morphological polarity and expressing brush-border hydrolases (Hidalgo, Raub, and Borchardt, 1989). Therefore, Caco-2 and HepG2 cell lines have been wildly used in various *in vitro* models, to investigate antioxidant activity of a compound toward the small intestinal epithelium and liver, respectively.

1.2 Research objectives

The objectives of this study were:

1. To produce protein hydrolysates with antioxidant properties from threadfin bream surimi byproducts, namely FBS and RD, using *Virgibacillus* sp. SK33 and commercial proteinases.

2. To fractionate and identify antioxidant peptides from FBS hydrolysates prepared from *Virgibacillus* sp. SK33 proteinase.

3. To fractionate FBS hydrolysates with antioxidant properties prepared from *Virgibacillus* sp. SK33 proteinase using ultrafiltration.

4. To determine pH and thermal stability as well as antioxidant activity after *in vitro* pepsin–pancreatin simulated GI digestion, of the active fraction.

1.3 Research hypotheses

Surimi byproducts have a potential as protein sources for production of protein

hydrolysates with antioxidant properties. *Virgibacillus* sp. SK33 proteinase can be used in production of protein hydrolysate with antioxidant properties. In addition, partially-purified peptides prepared from *Virgibacillus* sp. SK33 proteinases can act as radical scavenger, reducing power and metal chelator. Furthermore, ultrafiltrated fractions have different antioxidant activities compared to the parent hydrolysate, and can protect cell from oxidative damage-induced by TBHP. Antioxidant peptides are stable after pH and heat treatments and *in vitro* pepsin–pancreatin simulated GI digestion.

1.4 Scope of the study

FBS and RD were hydrolyzed with four proteinases; Alcalase, pepsin, trypsin and crude enzyme from *Virgibacillus* sp. SK33. Degree of hydrolysis (DH), amino acid composition, and antioxidant activity based on ABTS, FRAP, inhibition of β-carotene bleaching, and cytroprotective effect of protein hydrolysates were determined. FBS hydrolysate prepared from *Virgibacillus* sp. SK33 proteinase was purified using anion exchange and size-exclusion chromatography. Amino acid sequences were characterized by LC-MS/MS. Antioxidant activity of partially-purified peptides were tested using different chemical-based assays. Fraction showing the highest antioxidant activity was used for peptide synthesis and its antioxidant activity was tested. FBS hydrolysate was fractioned using ultrafiltration membranes with various molecular weight cut-off. Antioxidant activity of all fractions was investigated based on chemical and biological assays. pH and thermal stability as well as the activity after *in vitro* pepsin–pancreatin simulated GI digestion of active fraction were also determined.

1.5 Expected results

Results from this research will lead to utilization of surimi wastes and proteinase from a novel source, *Virgibacillus* sp. SK33. More knowledge about antioxidant peptide, especially its mode of action, will be gained. This research will also lead to more understandings about the cytoprotective effect of antioxidant peptides on the liver and intestinal cells. The resulting peptides would be a promising nutraceutical products.

1.6 References

Bamdad, F., Wu, J., and Chen, L. (2011). Effects of enzymatic hydrolysis on molecular structure and antioxidant activity of barley hordein. **Journal of Cereal Science.** 54: 20-28.

Carrasco-Castilla, J., Hernández-Álvarez, A. J., Jiménez-Martínez, C., Jacinto-Hernández, C., Alaiz, M., Girón-Calle, Vioque, J., and Dávila-Ortiz, G. (2012). Antioxidant and metal chelating activities of peptide fractions from phaseolin and bean protein hydrolysates. **Food Chemistry** 135: 1789-1795.

Chen, M., and Li, B. (2012). The effect of molecular weights on the survivability of casein-derived antioxidant peptides after the simulated gastrointestinal digestion. **Innovative Food Science and Emerging Technologies.** 16: 341-348.

Chen, H. M., Muramoto, K., Yamauchi, F., and Nokihara, K. (1996). Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. **Journal of Agricultural and Food Chemistry.** 44: 2619-2623.

Chow, C. K. (1988). Interrelationships of cellular antioxidant defense systems. In C. K. Chow (Ed.). In **Cellular Antioxidant Defense Mechanisms** (pp. 217-237). Boca Raton, FL: CRC Press.

Cinq-Mars, C. D., Hu, C., Kitts, D. D., and Li-Chan, E. C. Y. (2008). Investigations into inhibitor type and mode, simulated gastrointestinal digestion, and cell transport of the angiotensin I-Converting enzyme-inhibitory peptides in pacific hake (*Merluccius productus*) fillet hydrolysate. **Journal of Agricultural and Food Chemistry.** 56: 410-419.

Cornelli, U. (2009). Antioxidant use in nutraceuticals. **Clinics in Dermatology.** 27: 175-194.

Dávalos, A., Miguel, M., Bartolomé, B., and López-Fandiño, R. (2004). Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. **Journal of Food Protection**. 67: 1939-1944.

Grice, H. C. (1986). Safety evaluation of butylatedhydroxytoluene (BHT) in the liver, lung and gastrointestinal tract. **Food and Chemical Toxicology.** 24: 1127-1130.

Guenneugues, P. and Morrissey, M. T. (2005). Surimi resources. In J. W. Park (Ed.). **Surimi and Surimi seafood** (pp. 3-32). Boca Raton, FL: CRC Press.

Guo, H., Kouzuma, Y., and Yonekura, M. (2009). Structures and properties of antioxidative peptides derived from royal jelly protein. **Food Chemistry**. 113: 238-245.

Halliwell, B., Aeschbach, R., Loliger, J., and Aruoma, O.I. (1995). The characterization of antioxidants. **Food and Chemical Toxicology.** 33: 601-617.

Halliwell, B., Zhao, K., and Whiteman, M. (2000). The gastrointestinal tract: a major site of antioxidant action? **Free Radical Research**. 33: 819-830.

He, R., Girgih, A. T., Malomo, S. A., Ju, X., and Aluko, R. E. (2013). Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. **Journal of Functional Foods.** 5: 219-227.

Hidalgo, I. J., Raub, T. J., and Borchardt, R. T. (1989). Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. **Gastroenterology.** 96: 736-749.

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

Je, J. Y., Qian, Z. J., Lee, S. H., Byun, H. G., and Kim, S. K. (2008). Purification and antioxidant properties of bigeye tuna (*Thunnus obesus*) dark muscle peptide on free radical-mediated oxidative systems. **Journal of Medicinal Food.** 11: 629-637.

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

Klompong, V., Benjakul, S., Kantachote, D., Hayes, K. D., and Shahidi, F. (2008). Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from Alcalase and Flavourzyme. **International Journal of Food Science and Technology.** 43: 1019-1026.

Ktari, N., Jridi, M., Bkhairia, I., Sayari, N., Ben Salah, R., and Nasri, M. (2012). Functionalities and antioxidant properties of protein hydrolysates from muscle

of zebra blenny (*Salaria basilisca*) obtained with different crude protease extracts. **Food Research International.** 49: 747-756.

Kudo, K., Onodera, S., Takeda, Y. Benkeblia, N., and Shiomi, N. (2009). Antioxidative activities of some peptides isolated from hydrolyzed potato protein extract. **Journal of Functional Foods.** 1: 170-176.

Li, B., Chen, F., Wang, X., Ji, B., and Wu, Y. (2007). Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization–mass spectrometry. **Food Chemistry.** 102: 1135-1143.

Lima, C. F., Andrade, P. B., Seabra, R. M., Fernandes-Ferreira, M., and Pereira-Wilson, C. (2005). The drinking of a Salvia officinalis infusion improves liver antioxidant status in mice and rats. **Journal of Ethnopharmacology.** 97: 383-389.

Loguercio, C., and Federico, A. (2003). Oxidative stress in viral and alcoholic Hepatitis. **Free Radical Biology and Medicine.** 34: 1-10.

Mendis, E., Rajapakse, N., and Kim, S. K. (2005). Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. **Journal of Agricultural and Food Chemistry.** 53: 581-587.

Morrisey, M. T., Lin, J., and Ismond, A. (2005). Waste management and waste utilization. In J. W. Park (Ed.). **Surimi and surimi seafood** (second ed., pp. 279-323). New York: Marcel Dekker.

Morrissey, M. T. and Tan, S. M. (2000). World resources for surimi. In J.W. Park (Ed.). **Surimi and surimi seafood** (pp. 1-21). New York: Marcel Dekker.

Moure, A., Domínguez, H., and Parajó, J. C. (2006). Antioxidant properties of ultrafi-

ltration-recovered soy protein fractions from industrial effluents and their hydrolysates. **Process Biochemistry.** 41: 447-456.

Nalinanon, S., Benjakul, S., Kishimura, H., and Shahidi, F. (2011). Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. **Food Chemistry.** 124: 1354-1362.

Narayanan, V. S., Fitch, C. A., and Levenson, C. W., (2001). Tumor suppressor protein p53 mRNA and subcellular localization are altered by changes in cellular copper in human HepG2 cells. **Journal of Nutrition.** 131: 1427-1432.

Nawar, W. W. (1996). Lipids. In O. R. Fennema (Ed.). **Food chemistry** (pp. 225-319). New York: Marcel Dekker.

Ngo, D. H., Qian, Z. J., Ryu, B., Park, J. W., and Kim, S. K. (2010). *In vitro* antioxidant activity of a peptide isolated from Nile tilapia (*Oreochromis niloticus*) scale gelatin in free radical-mediated oxidative systems. **Journal of Functional Foods.** 2: 107-117.

Pangsorn, S., Laong-manee, P., and Siriraksophon, S. (2007). **Status of Surimi Industry in the Southeast Asia**. Southeast Asian Fisheries Development Center, Samutprakan, Thailand.

Pappenheimer, J. R. and Volpp, K. (1992). Transmucosal impedance of small intestine-correlation with transport of sugars and amino acids. **American Journal of Physiology.** 263: C480-493.

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

Raghavan, S., Kristinsson, H. G., and Leeuwenburgh, C. (2008). Radical scavenging and reducing ability of Tilapia (*Oreochromis niloticus*) protein hydrolysates. **Journal of Agricultural and Food Chemistry.** 56: 10359-10367.

Rajapakse, N., Mendis, E., Byun, H. G., and 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.

Saiga, A., Tanabe, S., and Nishimura, T. (2003). Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. **Journal of Agricultural and Food Chemistry.** 51: 3661-3667.

Samaranayaka, A. G. P. and Li-Chan, E. C. Y. (2011). Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. **Journal of Functional Foods.** 3: 229-254.

Schantz, M., Mohn, C., Baum, M., and Richling, E. (2010) Antioxidative efficiency of an anthocyanin rich bilberry extract in the human colon tumor cell lines Caco-2 and HT-29. **Journal of Berry Research.** 1: 25-33

Simmonds, N. J. and Rampton, D. S. (1993). Inflammatory bowel-disease-a radical view. **Gut.** 34: 865-868.

Sinsuwan, S., Rodtong, S., and Yongsawatdigul, J. (2010). A NaCl-stable serine proteinase from *Virgibacillus* sp. SK33 isolated from Thai fish sauce. **Food Chemistry.** 119: 573-579.

Sun, L., Zhang, Y., and Zhuang, Y. (2013). Antiphotoaging effect and purification of an antioxidant peptide from tilapia (*Oreochromis niloticus*) gelatin peptides. **Journal of Functional Foods.** 5: 154-162.

Vitaglione, P., Morisco, F., Caporaso, N., and Fogliano, V. (2004). Dietary antioxidant compounds and liver health. **Critical Reviews in Food Science and Nutrition.** 44: 575-586.

Wichi, H. P. (1988). Enhanced tumor development by butylated hydroxyanisole (BHA) from the oseophageal squamous epithelium. **Food and Chemical Toxicology.** 26: 717-723.

Xia, Y. C., Bamdad, F., Ganzle, M., and Chen, L. Y. (2012). Fractionation and characterization of antioxidant peptides derived from barley glutelin by enzymatic hydrolysis. **Food Chemistry.** 134: 1509-1518.

Zhang, J. H., Zhang, H., Wang, L., Guo, X. N., Wang, X. G., and Yao, H. Y. (2010). Isolation and identification of antioxidative peptides from rice endosperm protein enzymatic hydrolysate by consecutive chromatography and MALDI-TOF/TOF MS/MS. **Food Chemistry.** 119: 226-234.

Zhou, D. Y., Zhu, B. W., Qiao, L., Wu, H. T., Li, D. M., and Yang, J. F. (2012). In vitro antioxidant activity of enzymatic hydrolysates prepared from abalone (*Haliotis discus hannai Ino*) viscera. **Food and Bioproducts Processing.** 90: 148-154.

Zhu, K., Zhou, H., and Qian, H. (2006). Antioxidant and free radical-scavenging activities of wheat germ protein hydrolysates (WGPH) prepared with alcalase. **Process Biochemistry.** 41: 1296-1302.

CHAPTER II

LITERATURE REVIEWS

2.1 Surimi production, wastes and utilization

Surimi is stabilized fish myofibrillar proteins, which is prepared by water washing and blending with cryoprotectants (Park and Morrissey, 2000). Surimi production involves several processes including heading, gutting, deboning, mincing, washing, and refining processes. Minced fish is washed with chilled water and dewatered to produce high-quality surimi. The major objective of washing and dewatering is to remove soluble sarcoplasmic proteins, lipids, fish blood, and other water-soluble matters in the flesh. Connective tissues or refiner discharges (about 5% of the raw material) are separated in the refining process. Consequently, both soluble and solid wastes from surimi production are generated. Soluble proteins about 14-16% of the raw material are generated in wash water. Recovering sarcoplasmic proteins from waste water has been reported (Bourtoom, Chinnan, Jantawat, and Sanguandeekul, 2009; Kanjanapongkul, Yoovidhya, Tia, and Wongsa-Ngasri, 2008; Wibowo, Savant, Cherian, Savage, and Torres, 2005). Transglutaminase and trypsin inhibitors from threadfin bream sarcoplasmic proteins were have been recovered from surimi wash water by ultrafiltration (Piyadhammaviboon and Yongsawatdigul, 2009 and 2010). Solid wastes (viscera, heads, frames, skin, bones, and connective tissue) are about 50 to 70% of the original raw material. Over the past 50 years, there are several developments of solid waste utilization in the industry. Solid wastes can be

converted to pet food (Raa and Gildberg, 1982), fish protein hydrolysates (FPHs) (Benjakul and Morrissey, 1997), and fish meal (Kim and Park, 2005). Fish skins and bones (about 8-10% of the original raw material) are good source for gelatin production (Jamilah and Harvinder, 2002; Jongjareonrak, Benjakul, Visessanguan, and Tanaka, 2005). In addition, collagen was extracted from refiner discharges (Kim and Park, 2005). Heads and guts of threadfin bream, byproducts from surimi industry was used as a raw material for fish protein hydrolysate (FPH) production (Garnjanagoonchorn, Nitisinprasert, and Yampei, 1998). Proteinases from viscera (15-20% of the raw material) extract were already studied (Khantaphant and Benjakul, 2008).

Surimi production in Southeast Asia derived from Threadfin bream (*Nemipterus* sp.) and other tropical fish increased from 320,000 MT in 2005 to about 400,000 MT in 2011 (http://www.groundfishforum.com). Thailand is one of the largest surimi producers in Southeast Asia. Surimi production increased from 65,000 MT in 1978 to about 94,000 MT in 2011 (Pangsorn, Laong-manee, and Siriraksophon, 2007; http://www.groundfishforum.com). Fish used as a raw material of surimi production includes threadfin bream (*Nemipterus* spp.), bigeye snapper (*Priacanthus* spp.), croaker (*Pennahia* and *Johnius* spp.), lizardfish (*Saurida* spp.) and goatfish (*Upeneus* spp.) (Benjakul, Chantarasuwan, and Visessanguan, 2003). The quantity of fish supplied to the surimi industry was 530,000 MT in 2005 (Pangsorn et al., 2007). About 36% of surimi production was from threadfin bream, 18% of lizardfish and 17% of croaker (Pangsorn et al., 2007).

Threadfin bream is a major resource for surimi production with an approximate annual production of over 80,000 metric tons (Yongsawatdigul,

Worratao, and Park, 2002). This is because of its white color, good flavor and gel-forming ability. The quantity of threadfin bream raw materials supplied to surimi production is estimated to be 200,000 MT/year (Pangsorn et al., 2007). Normally, the solid wastes from deboning machines and a refining process are about 22–27% and 5%, respectively (Morrisey, Lin, and Ismond, 2005). It is, therefore, estimated that frame and skin/bone, as well as refiner discharges, from threadfin bream surimi production are about 50,000–60,000 MT annually. These wastes are usually converted to a low value fish meal. For this reason, a means to fully utilize and increase marketability of these wastes should be sought.

2.2 Free radicals and oxidative stress

Free radicals can be defined as a highly reactive molecule with one or more unpaired electrons in their outer orbit (Halliwell and Gutteridge, 1999; Mine and Katayama, 2008). Free radicals attempt to withdraw electrons from other molecule for their stability and start chain reactions, i.e. reactions that involve a number of steps, each of which forms a free radical that triggers the next step. There are three phases: initiation, propagation and termination (Buonocore, Perrone, and Tataranno, 2010). There are different free radical species, including oxygen-centered radicals (reactive oxygen species; ROS), nitrogen-centered radicals (reactive nitrogen species; RNS), carbon-centered radicals, and sulfur-centered radicals (Halliwell, 2007). ROS represent the most important class of radical species generated in living systems (Miller, Buettner, and Aust, 1990).

2.2.1 Reactive oxygen species (ROS)

ROS are divided into free oxygen radicals and non-radical ROS. Free oxygen

radicals are superoxide radical ($O_2^{\bullet-}$, and conjugated acid—hydroperoxyl radical, $HO_2^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), and alkoxy-($RO^{\bullet}$) or peroxyl-($ROO^{\bullet}$) radicals, as well as non-radical species that are oxidizing agents and/or easily converted into radicals, such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and lipid peroxide (LOOH) or hydroperoxide (ROOH) (Cornelli, 2009; Leonarduzzi, Sottero, and Poli, 2010).

ROS can be produced from both endogenous and exogenous substances. Potential endogenous sources include mitochondria, cytochrome P450 metabolism, neutrophils, eosinophils, macrophages, peroxisomes, microsomes, inflammatory cell activation, monoxygenase system, and several other enzymes involved in the inflammatory process, and other mechanisms (Authen and Davis, 2009; Cadenas, 1989; Conner and Grisham, 1996; Inoue, Sato, and Nishikawa, 2003; Valko, Izakovic, Mazur, Rhodes, and Telser, 2004; Valko, Rhodes, Moncol, Izakovic, and Mazur, 2006). ROS are also produced by a host of exogenous processes, such as environmental agents and xenobiotics (metal ions, radiations, barbiturates) (Klaunig, Xu, Bachowski, and Jiang, 1997).

$O_2^{\bullet-}$is the primary ROS, which generated by the process of reduction of molecular oxygen mediated by NAD(P)H oxidases and xanthine oxidase or non-enzymatically by redox-reactive compounds, such as the semi-ubiquinone compound of the mitochondrial electron transport chain (Valko et al., 2007). $O_2^{\bullet-}$ can further interact with other molecules to generate secondary ROS, like hydrogen peroxide (H_2O_2), hydroxyl radical and singlet oxygen (either directly or through enzyme- or metal-catalysed processes) (Bielski and Cabelli, 1991; Buonocore et al., 2010; Picardo and Passi, 1997; Valko et al., 2004; Valko, Morris, and Cronin, 2005). The production of various ROS is closely related with the participation of redox-active

metals (e.g. Fe^{2+}, Cu^{+} and others) (Valko et al., 2005). Under stress conditions, an excess of superoxide releases "free iron" from iron-containing enzymes (Liochev and Fridovich, 1994). The released Fe^{2+} can participate in the Fenton reaction to generate highly reactive $HO^{\bullet}$ (Valko et al., 2007). In addition, the superoxide radical participates in the Haber–Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + HO^{\bullet} + HO^{-}$) which combines a Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-}$) and the reduction of Fe^{3+} by superoxide, yielding Fe^{2+} and oxygen ($Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$) (Liochev and Fridovich, 2002).

ROS are well recognized for playing a dual role as both deleterious and beneficial species (Valko et al., 2006). Beneficial effects of ROS occur at low/moderate concentrations. Under physiological conditions, ROS are essential for life, and are involved in signal regulation, production of energy and defense mechanism against infection, and they play a physiological role as secondary messengers (Buonocore et al., 2010). On the other hand, the harmful effect of ROS causing potential biological damage is termed oxidative stress.

Oxidative stress is a highly oxidized environment within cells due to increased level of ROS over antioxidant capacity (Mine and Katayama, 2008). The excess ROS can damage cellular lipids, proteins, or DNA inhibiting their normal function leading to many serious diseases like cancers, inflammatory, the liver and Alzheimer's diseases, as well as aging (Balaban, Nemoto, and Finkel, 2005; Finkel, 2005; Hardy and Higgins, 1992; Kawanishi, Hiraku, Murata, and Oikawa, 2002; Loguercio and Federico, 2003; Simmonds and Rampton, 1993; Vitaglione, Morisco, Caporaso, and Fogliano, 2004). The harmful effects of ROS are balanced by the antioxidant action both antioxidant enzymes, and non-enzymatic antioxidants (Halliwell, 1996; Valko et

al., 2007).

Besides physiological effect, transition metals, such as Cu, Fe and Co, and free radicals affect both the rate of autoxidation and the direction of hydroperoxide breakdown to volatile compounds, causing rancidity, decrease in shelf life and the formation of potentially toxic reaction products (Nawar, 1996; Pihlanto, 2006). The –CH=CH–CH_2–CH=CH– structural elements of polyunsaturated fatty acids (PUFAs) are highly susceptible to attack by free radicals (Rajapakse, Mendis, Byun, and Kim, 2005a).

2.3 Antioxidant systems

In term of the effects in the human body, antioxidant can be defined as ‘‘a substance in foods that, significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen, on normal physiological functions” (Huang, Ou, and Prior, 2005). In term of foods, antioxidants are a substance that when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents autooxidation processes (Halliwell and Gutteridge, 1990; Mielnik, Aaby, and Skrede, 2003).

In the human body, under physiological conditions, cells are protected against autoxidation by antioxidant pathways, both enzymatic and non-enzymatic systems. The enzymatic systems include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Matés, Perez-Gomez, and Núñez de Castro, 1999). For antioxidant enzymes, superoxide dismutase (EC 1.15.1.1) catalyzes the dismutation of $O_2^{\bullet -}$ to O_2 and to the less-reactive species H_2O_2 (Fridovich, 1995; Matés and Sánchez-Jiménez, 1999). Catalase (EC 1.11.1.6) is a highly reactive enzyme, which converts

H_2O_2 to H_2O and O_2 (Matés and Sánchez-Jiménez, 1999). Glutathione peroxidase (EC 1.11.1.19) catalyses the reduction of a variety of hydroperoxides (ROOH and H_2O_2) using GSH as the electron donor, thereby protecting mammalian cells against oxidative damage and, reducing, among others, cellular lipid hydroperoxides (Jornot, Petersen, and Junod, 1998). The oxidized glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (Gred), which uses NADPH as the electron donor.

For non-enzymatic systems, the small-molecule antioxidants, such as vitamin C, vitamin E (α-tocopherol), thiol antioxidants (glutathione, thioredoxin, and lipoic acid), carotenoids, flavonoids, and ubiquinol act as radical scavenger,$^1O_2$ quenchers, hydrogen and/or electron donors (Chow, 1988; Picardo and Passi, 1997). α-Tocopherol is a fat-soluble vitamin. Its major antioxidant action in biological membranes is to act as a chain breaking antioxidant, donating labile hydrogen to peroxy and alkoxy radicals, thereby breaking the radical chain. It has been proposed that α-tocopherol radical can be reduced to the original tocopherol form by ascorbic acid or reduced glutathione (Kojo, 2004; Packer, Slater, and Wilson, 1979). Vitamin C (ascorbic acid) is a very important and powerful antioxidant that works in aqueous environments of the body. Vitamin C can act as a radical scavenger. In addition, there are proteins, such as transferrin, ferritin, lactoferrin and lactalbumin that can chelate iron, or ceruloplasmin and albumin that can bind to copper (Chow, 1988). The activities and the intracellular levels of enzymatic and non-enzymatic antioxidants are very important aspect for survival of living organisms and health.

In term of foods, synthetic antioxidants, such as 3-ter-butyl-4-hydroxyanisole (BHA), 3, 5-di-tert-butyl-4-hydroxytoluene (BHT), tertiary-butylhydroxyquinone

(TBHQ) and propyl gallate, are commonly used in food industry to retard lipid oxidation. However, BHA and BHT are likely to cause liver damage and carcinogenesis (Grice, 1986; Wichi, 1988). It is, therefore, of great interest both from academic and practical points of view in exploring alternative antioxidants from many natural sources that enhance antioxidative defenses in the body and retard lipid oxidation in food systems.

2.4 Bioactive peptides

Recently, interest has been paid to identify and characterize bioactive peptides from plant and animal sources (Sarmadi and Ismail, 2010). Bioactive peptides are considered as specific protein fragments that have a positive impact on body's function and health (Kitts and Weiler, 2003). Bioactive peptides are inactive within the sequence of the parent protein, but can be released during gastrointestinal digestion, during food processing or by enzymatic hydrolysis (Lahl and Braun, 1994; Vercruysse, Van camp, and Smagghie, 2005). These peptides usually contain 2-20 amino acid residues, and molecular weight of less than 6000 Da (Meisel and FitzGerald, 2003; Sun, He, and Xie, 2004). Based on their structural properties and their amino acid composition and sequences, they may be involved in various biological functions, such as antihypertension, opioid, immunomodulatory, antithrombotic, antioxidant, anti-cancer, and antimicrobial activities, in addition to nutrient utilization (Elias, Kellerby, and Decker, 2008; Liu et al., 2008). One of the most crucial functions of bioactive peptides is the antioxidant activity, because its beneficial effects are related to many diseases (Gu et al., 2012).

2.4.1 Protein hydrolysates and antioxidant peptides

2.4.1.1 Enzymatic hydrolysis

Protein hydrolysate can be defined as a mixture that is mainly composed of peptides and amino acids which are produced through protein hydrolysis by enzyme (exogenous proteolytic enzymes, autolytic process using endogenous enzymes), acid or alkaline treatment or microbial fermentation (Samaranayaka, Kitts, and Li-chan, 2010; Sarmadi, and Ismail, 2010). Among these methods, use of exogenous enzymes is widely applied process for the production of protein hydrolysates or peptides with antioxidant properties because of the shorter time required to obtain similar degree of hydrolysis as well as better control of the hydrolysis to obtain more consistent molecular weight profiles and peptide composition (Samaranayaka and Li-chan, 2011).

Proteinases from different sources, including animal viscera, plants and microorganisms are commonly employed for production of protein hydrolysate. Commercial proteinases used for the production of protein hydrolysates with antioxidant properties are Alcalase, Flavourzyme, Neutrase, pepsin trypsin, chymotrypsin, pancreatin, papain, and bromelain (Je, Qian, Byun, and Kim, 2007).

Alcalase is a commercial proteinase from a microbial source, namely *Bacillus licheniformis* and is shown to be one of the most efficient enzymes for protein hydrolysis (Guérard, Dufossé, De La Broise, and Binet, 2001). In addition, in comparison to other proteinases, Alcalase provides higher yields of antioxidant peptides and produced shorter peptide, which are more resistant to digestive enzymes (Kim et al., 2001; Park, Jung, Nam, Shahidi, and Kim, 2001). Alcalase is an endoproteinase cleaves peptide bonds at the interior of the polypeptides chain; thus, it

mainly produces small- and medium-sized peptides (Klompong, Benjakul, Kantachote, Hayes, and Shahidi, 2008). Alcalase shows broad specificity, and high specificity toward aromatic (Phe, Trp, and Tyr), acidic (Glu), sulfur-containing (Met), aliphatic (Leu and Ala), hydroxyl (Ser), and basic (Lys) residues (Doucet, Otter, Gauthier, and Foegeding, 2003). Alcalase-derived peptide from silver carp showed higher chelating activity than Flavourzyme at all hydrolysis times (Dong et al. 2008). Flavourzyme contains both exopeptidases and endoproteinases, and has broad specificity to produce small-size peptides and free amino acids (Van der Ven, Gruppen, de Bont, and Voragen, 2002). Flavourzyme has been used for the production of antioxidant peptides from protein of different sources, such as round scad (Thiansilakul, Benjakul, and Shahidi 2007), and tilapia (Raghavan, Kristinsson, and Leeuwenburgh, 2008). Neutrase is produced from *Bacillus amyloliquefaciens* and is a metalloendoproteinase which prefers to catalyze hydrophobic amino acids. The enzyme has many catalyzing sites (Zhao and Hou, 2009). Neutrase was the most effective for production antioxidant peptides from rice endosperm protein (Zhang et al., 2010). Gastrointestinal enzymes (pepsin, trypsin, chymotrypsin and pancreatin)-derived peptide showed scavenging on hydroxyl radical (Byun et al. 2009; You, Zhao, Regenstein, and Ren, 2010). Pepsin shows specificity toward aromatic amino acids: Phe, Tyr, and Trp, and Leu and Glu at the carboxyl side of a peptide bond (Simpson, 2000; You et al., 2010). Therefore, hydrolysis with pepsin generates peptides containing Tyr, Phe, or Leu in the N-terminal positions (Savoie, Gauthier, Marin, and Pouliot, 2005). Trypsin is specific for Lys or Arg at the carboxyl side of a peptide bond (Brown and Wold, 1973; Folk and Schirmer, 1965). α-Chymotrypsin hydrolyzes proteins at the carboxyl side of Leu, Tyr, Phe, Trp, and Met residues (Robyt and

White, 1987). Pancreatin contains many enzymes, including trypsin, α-chymotrypsin, and elastase and exopeptidases of carboxypeptidases A and B, with trypsin and chymotrypsin predominantly responsible for the majority of digestion in the duodenum (Young, Nau, Pasco, and Mine, 2011). Hydrolysates produced by pancreatin are high in hydrophobic amino acid residues, such as Val, Leu, and Phe (Mullally, Ocallaghan, Fitzgerald, Donnelly, and Dalton, 1994). Pancreatin has been used for the production of antioxidant peptides from potato protein (Kudo, Onodera, Takeda, Benkeblia, and Shiomi, 2009). The use of gastrointestinal proteinases produces antioxidant peptides that are resistant to the physiological digestion (Qian**,** Jung, Byun, and Kim**,** 2008).

Papain from papaya is a cysteine protease containing endopeptidases, aminopeptidases, dipeptidyl peptidases and enzymes with both exo- and endo-peptidase activity (Rawlings and Barrett, 1994). Papain was the most effective for production of protein hydrolysate with antioxidant activity from venison proteins (Kim et al., 2009). Bromelain from pineapple stemis a cysteine protease which prefers to cleave at Lys, Ala, Tyr and Gly (Yang, Ho, Chu, and Chow, 2008). Grass carp muscle hydrolysate prepared by bromelain showed the lowest antioxidant activity (Ren et al., 2008).

Besides commercial proteinases, the use of proteinases from novel sources in protein hydrolysate production has not been widely studied. Crude proteinase extracted from mackerel intestine (MICE) has been used for production of antioxidant peptide from yellowfin sole frame and Alaska pollack frame protein (Je, Park, and Kim, 2005; Jun, Park, Jung, and Kim, 2004). In addition, antioxidant peptides from sardinelle byproducts can be produced by crude enzyme extract from viscera of

sardine (Bougatef et al., 2010). Using different enzymes to produce protein hydrolysates resulted in the formation of a mixture of peptides with different sizes, and amino acid sequences, resulting in various antioxidant activities (Chen, Muramoto, and Yamauchi, 1995; Jeon, Byun, and Kim, 1999; Wu, Chen, and Shiau, 2003).

Sinsuwan, Rodtong, and Yongsawatdigul (2008) reported that extracellular proteinases from *Virgibacillus* sp. SK33, isolated from one month-old Thai fish sauce mashes exhibited optimum activity at 50°C and pH 8-11 with molecular weight of 56, 46, 42, 32, 25 and 19 kDa. In addition, *Virgibacillus* sp. SK33 proteinases showed the highest catalytic activity of subtilisin-like alkaline serine proteinase characteristic and hydrolyzed anchovy protein to a greater extent than did Alcalase at the same unit. The purified *Virgibacillus* sp. SK33 proteinases showed broad specificity toward oxidized insulin B including Gln^{4}, Cys^{7}, Glu^{13}, Ala^{14}, $Leu^{15,17}$, $Tyr^{16,26}$, Arg^{22}, $Phe^{24,25}$, and Lys^{29} (Sinsuwan, Rodtong, and Yongsawatdigul, 2010). *Virgibacillus* sp. SK33 proteinases could be a potential processing-aid for production of bioactive protein hydrolysate with antioxidant activity.

2.4.1.2 Sources of protein hydrolysates and/or antioxidant peptides

Recently, researchers have started focusing their attention on the nutraceutical properties of protein hydrolysates from various sources including animals and plants. It seems that in the area of nutritional sciences, application of non-purified protein hydrolysate can have certain benefits over those of purified peptides since the absorption of oligopeptides can be increased in the presence of sugar and amino acids (Pappenheimer and Volpp, 1992). Moreover, a critical step toward commercializing fish protein hydrolysates as a nutraceutical product would involve

evaluation of the antioxidant properties of the hydrolysate as a whole, rather than identifying individual peptide (Raghavan et al., 2008). The utilization of protein hydrolysates or peptides as antioxidant in foods presents additional advantages over other natural antioxidants since they also conferred nutritional value as well as desired functional properties (Gómez-Ruiz, López-Expósito, Pihlanto, Ramos, and Recio, 2008). In addition, antioxidant peptides derived from foods are considered to be safe and healthy compounds with low molecular weight, low cost, high activity, and easy absorption (Sarmadi and Ismail, 2010).

Protein hydrolysates with antioxidant activity derived from enzymatic hydrolysis of plant sources include soybean (Moure, Domínguez, and Parajó, 2006), zein (Kong and Xiong, 2006), wheat germ (Zhu, Zhou, and Qian, 2006), alfalfa leaf (Xie, Huang, Xu, and Jin, 2008), canola (Cumby, Naczk, Zhong, and Shahidi, 2008), chickpea (Li, Jiang, Zhang, Mu, and Liu, 2008), buckwheat (Tang, Peng, Zhen, and Chen, 2009), cotton leafworm (Vercruysse, Smagghe, Beckers, and Camp, 2009), peanut (Jamdar et al., 2010), Amaranth (Tironi and Anón, 2010) and barley hordein (Bamdad, Wu, and Chen, 2011).

In addition, antioxidant peptides derived from enzymatic hydrolysis of animals and animal products are egg yolk (Sakanaka and Tachibana, 2006; Sakanaka, Tachibana, Ishihara, and Juneja, 2004), milk (Hogan, Zhang, Li, Wang, and Zhou, 2009; Rival, Boeriu, and Wichers, 2001), whey (Peña-Ramos and Xiong, 2001; Peng, Xiong, and Kong, 2009), porcine hemoglobin (Chang, Wu, and Chiang, 2007), porcine plasma (Liu, Kong, Jiang, Cui, and Liu, 2009), and chicken (Liu et al., 2011).

Aquatic products and byproducts have also been reported to be a good source of antioxidant peptides, such as mackerel (Wu et al., 2003), jumbo flying squid (Lin

and Li, 2006), round scad (Thiansilakul et al., 2007), yellow stripe trevally (Klompong et al., 2008), brownstripe red snapper (Khantaphant and Benjakul, 2008), silver carp (Dong et al., 2008), tilapia (Raghavan et al., 2008), smooth hound (Bougatef et al., 2009), tuna liver (Je, Lee, Lee, and Ahn, 2009), loach (You et al., 2010), Black scabbardfish (Batista, Ramos, Coutinho, Bandarra, and Nunes, 2010), Pacific abalone (Zhou et al., 2012). Antioxidant activity of those hydrolysates varied with proteinase used, degree of hydrolysis, hydrophobicity, molecular weight (Mw) distribution, and amino acid composition.

2.4.1.3 Purified antioxidant peptides

Amino sequencing and characterization of individual peptides could be important for studying mechanism and may provide information about characteristics of bioactive peptide (Raghavan et al., 2008). In addition, amino acid sequences will be useful for the peptide synthesis for either food or pharmaceutical industry. Several studies isolated and identified the compounds, which exhibit antioxidant properties using membrane filtration, chromatography and mass spectrometry (Chen, Yang, Sun, Niu, and Liu, 2012). And these compounds were normally tested for inhibition of lipid oxidation, radicals scavenging, and metal chelating ability. List of purified antioxidant peptide derived from various sources is shown in Table 2.1. Kim et al. (2001) reported that peptides derived from Alaska pollack skin showed both antioxidative activity and synergistic effect with α-tocopherol using the linoleic acid in water/alcohol system. Furthermore, the authors also suggested that isolated antioxidative peptide composing amino acid sequences of GPOGPOGPOGPOG showed higher antioxidant property than those with the sequence of GEOGPOGPOGPOGPOG. They concluded that antioxidant activity of peptides

derived from Alaska pollack skin depended on their amino acid sequences. Mendis, Rajapakse, Byun, and Kim (2005a) reported that jumbo squid skin gelatin peptides exhibited a strong lipid peroxidation inhibition and it was much higher than that of natural antioxidant, α-tocopherol. In addition, it could scavenge highly active free radicals in oxidative systems ($^{\bullet}OH$, $R^{\bullet}$, $RO^{\bullet}$ and $ROO^{\bullet}$). Two purified peptides; FDSGPAGVL (880.18 Da) and NGPLQAGQPGER (1241.59 Da), exhibited the highest activity. Furthermore, Rajapakse et al. (2005a) also reported that two peptides from giant squid muscle composed of 7 and 13 amino acid residues exhibited their antioxidant potential as chain-breaking antioxidants by inhibiting radical-mediated peroxidation of linoleic acid, and their activities were similar to highly active BHT.

Je et al. (2005) reported that Alaska pollack frame protein hydrolysate with molecular weight below 1 kDa exhibited the highest antioxidative activity on linoleic acid model system. In addition, the purified peptide (LP**H**SG**Y**; 672 Da) scavenged $^{\bullet}OH$. The authors suggested that this activity may be attributed to the chelating and lipid radical-trapping ability of the imidazole ring. Furthermore, this peptide contained tyrosine residue which is a potent hydrogen donor.

Kim, Je, and Kim (2007) reported that purified peptide from hoki frame protein hydrolysate had a molecular mass of 1801 Da, and amino acid sequence was identified as ESTVPERTHPACPDFN. The peptide exhibited the inhibition of lipid peroxidation higher than α-tocopherol, and efficiently quenched DPPH, $^{\bullet}OH$, $O_2^{-\bullet}$, $R^{\bullet}$, $RO^{\bullet}$ and $ROO^{\bullet}$ radicals. The authors suggested that antioxidant peptides from hoki frame protein hydrolysate composed of 44% hydrophobic amino acids exerting a high affinity to linoleic acid by interacting with lipid molecules and donating its protons to lipid-derived radicals.

Table 2.1 Purified antioxidant peptide derived from various sources.

Source of peptides	Sequence	Preparation	Activity	Reference
1. Fish				
Alaska pollack skin gelatin	GEOGPOGPOGPOGPOG and GPOGPOGPOGPOG	Serial digestions in the order of Alcalase, Pronase E	Inhibition of linoleic acid autoxidation and protect Donryu rat liver cells from oxidant injury by TBHP	Kim et al. (2001)
Yellowfin sole (*Limanda aspera*) frame	RPDFDLEPPY	Pepsin followed Mackerel intestines crude enzyme (MICE)	Inhibition of linoleic acid autoxidation	Jun et al. (2004)
Alaska Pollack frame	LPHSGY	Mackerel intestine crude enzyme (MICE)	Inhibition of linoleic acid autoxidation and hydroxyl radical-scavenging activity	Je, Park, and Kim (2005)
Conger eel muscle	LGLNGDDVN	Trypsin	Inhibition of lipid peroxidation, hydroxyl, and peroxyl radical scavenging activity	Ranathunga, Rajapakse, and Kim (2006)
Tuna backbone	VKAGFAWTANQQLS	Pepsin	Inhibition of linoleic acid autoxidation , DPPH, hydroxyl and superoxide radical-scavenging activity	Je et al. (2007)
Hoki (*Johnius belengerii*) frame	ESTVPERTHPACPDFN	Pepsin	Inhibition of linoleic acid autoxidation, DPPH, hydroxyl, superoxide and peroxyl radical-scavenging activity, protect MRC-5 cells from oxidant injury by TBHP and protective effects on DNA damage	Kim et al. (2007)
Bigeye tuna dark muscle by-products	LNLPTAVYMVT	Pepsin	Inhibition of linoleic acid autoxidation, DPPH, hydroxyl, superoxide, peroxyl radical scavenging activity	Je, Qian, Lee, Byun, and Kim (2008)
Grass carp muscle	PSKYEPFV	Alcalase 2.4L	Inhibition of rat liver lipid oxidation and hydroxyl radical scavenging activity	Ren et al. (2008)

Table 2.1 (Continued).

Source of peptides	Sequence	Preparation	Activity	Reference
Tuna dark muscle by-products	LPTSEAAKY from OR and PMDYMVT from PR	Orientase (OR) and protease XXIII (PR)	Inhibition of linoleic acid autoxidation and DPPH radical-scavenging activity	Hsu (2010)
Nile tilapia (*Oreochromis niloticus*) scale gelatin	DPALATEPDPMPF	Alcalase	DPPH, superoxide and hydroxyl radical-scavenging activity, prevention of cellular and protective effects on DNA damage	Ngo, Qian, Ryu, Park, and Kim (2010)
Loach	PSYV	Papain	Inhibition of linoleic acid autoxidation, DPPH and hydroxyl radical-scavenging activity, and Copper-chelating activity	You et al. (2010)
Sardinelle (*Sardinella aurita*) by-products	LHY, LARL, GGE, GAH, GAWA, PHYL and GALAAH, and LHY was the most effective	Crude enzyme extract from sardine viscera (CESV)	Inhibition of linoleic acid autoxidation, reducing power and DPPH radical-scavenging activity	Bougatef et al. (2010)
Horse mackerel(*Magalaspis cordyla*) viscera	ACFL	Pepsin followed by trypsin and α-chymotrypsin	Inhibition of linoleic acid autoxidation and hydroxyl- and DPPH radical-scavenging activity.	Sampath Kumar, Nazeer, and Jaiganesh (2011)
Tilapia (*Oreochromis niloticus*) skin gelatin	EGL and YGDEY	Multifect neutral and followed by properase E	DPPH radical, superoxide anion radical (•O2) and hydroxyl radical (•OH) scavenging activities	Zhang, Duan, and Zhuang, (2012)
Pacific cod skin gelatin	LLMLDNDLPP	Pepsin followed by trypsin and α-chymotrypsin	Hydroxyl radical scavenging, Reduced the oxidation levels of membrane lipids, proteins and DNA in RAW264.7 cells,effectively scavenging the intracellular ROS and upregulated the m-RNA	Himaya, Ngo, Ryu and Kim (2012).

Table 2.1 (Continued).

Source of peptides	Sequence	Preparation	Activity	Reference
			expression of cellular antioxidative enzymes (SOD, GPx and CAT)	
Flounder fish *(Paralichthys olivaceus)*	VCSV and CAAP	α-Chymotrypsin	DPPH, hydroxyl and peroxyl radicals scavenging activity, cytoprotective activities against 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH), scavenged ROS in Vero cells and reduced apoptotic bodies produced by AAPH	Ko et al. (2013)
2. Other aquatic animals				
Jumbo squid (*Dosidicus gigas*) skin gelatin	FDSGPAGVL and NGPLQAGQPGER	Trypsin	Inhibition of linoleic acid autoxidation, hydroxyl and peroxyl radical-scavenging activity, and protect MRC-5 cells from oxidant injury by TBHP	Mendis et al. (2005)
Giant squid (*Dosidicus gigas*) muscle	NADFGLNGLEGLA and NGLEGLK	Trypsin	Inhibition of linoleic acid autoxidation, hydroxyl, superoxide and peroxyl radical-scavenging activity, and protect MRC-5 cells from oxidant injury by TBHP	Rajapakse et al. (2005a)
Fermented marine blue mussel (*Mytilus edulis*) sauce	HFGBPFH	Autolysis	Inhibition of lipid peroxidation, DPPH, hydroxyl, superoxide, peroxyl radical-scavenging activity, iron-chelating activity and protect MRC-5 cells from oxidant injury by TBHP	Rajapakse et al. (2005b)
Fermented marine blue mussel (*Mytilus edulis*) sauce	FGHPY	Autolysis	Inhibition of linoleic acid autoxidation and hydroxyl radical-scavenging activity	Jung, Rajapakse, and Kim (2005)

Table 2.1 (Continued).

Source of peptides	Sequence	Preparation	Activity	Reference
Bullfrog (*Rana catesbeiana Shaw.*) skin	LEELEEELEGCE	Alcalase	Inhibition of lipid peroxidation, DPPH, hydroxyl, superoxide, peroxyl radical scavenger	Qian, Jung, and Kim (2008b)
Oyster (*Crassostrea gigas*)	LKQELEDLLEKQE	Pepsin followed by trypsin and α-chymotrypsin	Inhibition of autooxidation, superoxide and hydroxyl radical-scavenging activity, prevention of cellular and protective effects on DNA damage	Qian et al. (2008a)
Marine rotifer, *Brachionus rotundiformis*	LLGPGLTNHA and DLGLGLPGAH	Pepsin	DPPH radical scavenging activity	Byun et al. (2009)
Blue mussel (*Mytilus edulis*)	YPPAK	Neutrase	Inhibition of linoleic acid autoxidation, DPPH, hydroxyl, and superoxide radical scavenging activity	Wang et al. (2013)
3. Animal and animal products				
Bovine liver sarcoplasmic	FGKEFTPVLQADFQK FGDLSTADAVMNNPK LHVDPENFKL VLSAADKGNVKA FSDVHPEYGSR AQKPDVLTTGGGNPV GDKLNS AAQKPDVLTTGGGNP VGDKLNS LVQDVVFTDEMAH VGMPDDIIQKGKD	Thermolysin	DPPH radical scavenging activity, reducing power (FRAP assay) and Fe^{2+}-chelating activity	Di Bernardini et al. (2011)
Hen egg white lysozyme	NTDGSTDYGILQINSR	The mixture of trypsin and papain	DPPH, and ABTS radical scavenging activity, Fe^{2+}-chelation ability, inhibition of linoleic acid peroxidation	Memarpoor-Yazdi, Asoodeh and Chamani (2012)

Table 2.1 (Continued).

Source of peptides	Sequence	Preparation	Activity	Reference
4. Plant				
β-conglycinin (7s protein)	VNPHDHQN LVNPHDHQN LLPHH LLPHHADADY VIPAGYP LQSGDALRVPSGTTYY	Protease S	Inhibition of linoleic acid peroxidation	Chen et al. (1995)
Soybean	His-containing peptides	Synthetic peptides were designed on the basis of the antioxidative peptide (LLPHH) derived from protease S digests of a soybean protein	Metal-ion chelator, an active-oxygen quencher and hydroxyl radical scavenger	Chen et al. (1998)
Corn gluten	FPLEMMPF	Alcalase	Inhibition of pyrogallol autoxidation	Zheng et al. (2006)
Algae protein waste	VECYGPNRPQF	Pepsin	Hydroxyl, superoxide, peroxyl, DPPH and ABTS radicals scavenging activity, protective effects on DNA and prevention of cellular damage	Sheih, Wu, and Fang (2009b)
Potato protein	FGER, FDRR and FGERR	The mixture of Pancreatin and Amano-P	Inhibition of linoleic acid peroxidation, inhibition of lipid oxidation in the erythrocyte membrane ghost and reduced ethanol-induced gastric mucosal damage	Kudo et al. (2009)
Rice endosperm protein	FRDEHKK and KHDRGDEF	Neutrase	Inhibition of lipid peroxidation, hydroxyl, DPPH, superoxide, and radical scavenging activity, protect MRC-5 and	Zhang et al. (2010)

Table 2.1 (Continued).

Source of peptides	Sequence	Preparation	Activity	Reference
			RAW264.7 cells from oxidant injury by TBHP, and prevention of cellular damage	
Chickpea (*Cicer arietium L.)*	NRYHE	Alcalase 2.4 L FG	DPPH, hydroxyl, and superoxide free radicals, Fe^{2+}- and Cu^{2+}-chelation ability, inhibition of linoleic acid peroxidation	Zhang, Li, Miao, and Jiang (2011)
Walnut (*Juglans regia* L.)	ADAF	Pepsin	Quench the hydroxyl radical, chelate ferrous ion, exhibit reducing power and inhibit the lipid peroxidation.	Chen et al. (2012)
Whey	VHLKP	Alcalase	Reducing power, inhibiting H_2O_2-induced oxidative damage in MRC-5 cells by neutralize radicals and other ROS, elevating the activity of SOD, CAT, GSH-Px and decreased the level of malonaldehyde (MDA)	Kong, Peng, Xiong, and Zhao (2012)
Chickpea (Cicer arietinum L.) albumin	RQSHFANAQP	Sequentially using Alcalase and Flavorzyme	Reducing power, DPPH, TEAC, and hydroxyl radical scavenging activity	Kou et al. (2013)
Zizyphus jujube fruits	VGQHTR and GWLK	Trypsin	DPPH, and ABTS radical scavenging activity, Fe^{2+}-chelation ability, reducing power and inhibition of linoleic acid peroxidation	Memarpoor-Yazdi, Mahaki and Zare-Zardini (2013)

2.4.2 Factor affecting antioxidant activity of protein hydrolysate or peptides

Antioxidant activity of protein hydrolysates is influenced by, degree of hydrolysis (You, Zhao, Cui, Zhao, and Yang, 2009), peptide size or molecular weight (Mw), amino acid composition, sequence, and peptide configuration (Chen et al., 1996; Xia, Bamdad, Ganzle, and Chen, 2012), protein substrate (Bougatef et al., 2010), and type of proteinase (Aleman, Gimenez, Montero, and Gomez-Guillen, 2011; Hernández-Ledesma, Davalos, Bartolome, and Amigo, 2005; Zhou et al., 2012).

2.4.2.1 Degree of hydrolysis (DH)

DH greatly influences the peptide chain length as well as the exposure of the terminal amino groups (Thiansilakul et al., 2007). In general, antioxidant activity increased with an increase in the DH (Phanturat, Benjakul, Visessanguan, and Roytrakul, 2010; Raghavan et al., 2008). Since high DH results in high small peptides, increasing peptide solubility. The increase of radical-scavenging activity of hydrolysates with higher DH may be attributed to the increasing hydrogen donating properties of the active peptides, which could react with free radicals (Batista et al., 2010). In contrast, the lack of a direct relationship between antioxidant activity and DH suggested that the specific composition (e.g., type of peptides, ratio of different free amino acids) was an important factor as well (Kong and Xiong, 2006; Pihlanto, 2006).

2.4.2.2 Molecular weight

Many researchers reported that low molecular weight peptides showed high antioxidant activity (Hernandez-Ledesma et al., 2005; Mendis et al., 2005b;

Rajapakse et al. 2005a). However, there is a lack of evidence to suggest which molecular weight range encompasses the most potent peptides (Cheung, Cheung, Tan, and Li-Chan, 2012). Some researchers reported that peptides with mass 1-3 kDa showed the highest antioxidant activities (Kim et al., 2007), while some reported that peptides with molecular weight lower than 3.5 kDa possessed a stronger antioxidant activity (Bougatef, Hajji, Balti, Lassoued, Triki-Ellouz, and Nasri, 2009). In addition, the small-size peptides with molecular weight ranging from approximately 0.2-6 kDa probably contributed to higher antioxidant activity (Liu et al., 2011). In contrast, the peptide with molecular weight of approximately 1.4 kDa possessed a stronger *in vitro* antioxidant activity than that of the 0.9 and 0.2 kDa peptides (Wu et al., 2003). Hwang, Shyu, Wang, and Hsu (2010) reported that the esperase hydrolysate of peanut protein with molecular weight 3-5 kDa showed higher relative antioxidative activity than that with molecular weight lower than 3 kDa or higher than 5 kDa. These suggested that peptide size might not be the most important factor, amino acid composition and peptide sequences might play a more crucial role in antioxidant activity (Cheung et al., 2012).

2.4.2.3 Amino acid composition

Antioxidant peptides derived from different sources have exhibited varying potencies to scavenge free radicals. But, the exact mechanism of scavenging these radicals is not clearly understood. Peptides containing aromatic amino acids (Tyr, His, Trp and Phe) can make reactive oxygen species (ROS) stable through direct electron transfer and maintaining their stability via resonance structures (Qian et al., 2008b; Rajapakse et al., 2005b). In addition, Trp and Tyr contain the indolic and phenolic groups, respectively, which serve as hydrogen donors. It is, therefore, likely

that oxygen radical quenches the Trp-indolic and Tyr-phenolic hydrogen (H^+), resulting in the formation of more stable indoyl and phenoxyl radicals (Hernández-Ledesma, et al., 2005). His contains an imidazole ring which may be involved in hydrogen donation and lipid radical trapping ability (Chen, Muramoto, Yamauchi, and Nokihara, 1996).

Met is prone to oxidation to Met sulfoxide and Cys donates the sulfur hydrogen and can directly interact with radical. Furthermore, Gly, Asp and Glu are able to quench unpaired electrons or radicals by supporting protons (Qian et al., 2008b). Peptides containing acidic (Asp and Glu) and basic (His, Lys and Arg) amino acids with carboxyl and amino groups in the side chains are thought to play an important role in chelating metal ions (Rajapakse et al., 2005a; Saiga, Tanabe, and Nishimura, 2003); Zhang et al., 2010). Peptides containing hydrophobic amino acids may contribute to inhibition of lipid peroxidation since they can increase the solubility of peptides in the lipid system and thereby facilitating better interaction with the radical species (Chen et al., 1996). Chen et al. (1998) reported that histidine-containing peptides derived from the digests of soybean protein could not interact properly with hydrophobic peroxyl radicals due to lack of hydrophobicity in peptides.

2.4.2.4 Amino acid sequence

The positioning of amino acids in the peptide sequence plays a vital role in antioxidant activity. Phe and Leu residues at N- and C-terminal of **F**DSGPAGV**L** contributed to the antioxidant activity (Mendis et al., 2005a). N-terminus histidine residue of **H**GPLGPL can be expected as a strong proton-donating residue in the sequence (Mendis, Rajapakse, and Kim, 2005b). Leu is important for the antioxidant activity of a peptide when present at the C-terminus (Mendis et al., 2005b; Suetsuna,

Ukeda, and Ochi, 2000). Pro is also important for the antioxidant activity of a peptide when present at the C-terminus (Suetsuna et al., 2000). Moreover, many peptides that have hydrophobic amino acid residues such as, Val, Leu, Ile, or Ala, at their N-terminal positions (Guo, Kouzuma, and Yonekura, 2009; Kawashima, Itoh, Miyoshi, and Chibata, 1979; Suetsuna et al., 2000) and peptides that have Lys at their N-termini showed strong antioxidative activity. The authors suggested that it is probable that these amino acid residues play important antioxidative roles. Glu-Leu (EL) sequence is important for the superoxide radical scavenging activity (Suetsuna et al.). Rajapakse et al. (2005a) suggested that Gly-Leu (GL) sequence was frequently observed in NADF**GL**N**GL**E**GL**A (1307 Da) and N**GL**E**GL**K (747 Da) of giant squid muscle which were expected to favor inhibition of oxidation. Kim et al. (2001) reported that the difference of antioxidative activity between the two peptides, **GEO**GPOGPOGPOGPOG and GPOGPOGPOGPOG isolated from Alaska Pollack Skin gelatin is thought to be attributable to the first three amino acid residues (GEO) at N-terninus. Sadat et al. (2011) reported that five peptides (**Y**DTQA, IN**Y**, LDQ**W**, INY**W** and VSLPE**W**) from α-lactalbumin, all containing at least one Tyr or Trp residue located at one of the extremities of the sequence, displayed the most efficient radical scavenging activity. Moreover, they also reported that radical scavenging activity of YGGVSLPEW were 62% at 100 μM. However, YGGVSLPEW was further hydrolyzed into two final peptides, YGG and VSLPEW, which showed higher radical scavenging activity (75% at 100 μM and 100% at 25 μM, respectively) than those YGGVSLPEW. This result confirmed influence of amino acid sequence.

2.4.2.5 Peptide bone or configuration

Antioxidant activity of peptide is influenced by the peptides configuration.

Chen et al. (1996) reported that substitution for the second L-His with D-His diminished the activity. The authors suggested that the correct positioning of imidazole groups is essential for the antioxidant activity. In addition, peptide bond or conformation and/or specific structural features of the peptides have been reported to influence antioxidant activity. Certain amino acids can exert higher antioxidant activities when they are incorporated in dipeptides (Nagasawa, Yonekura, Nishizawa, and Kitts, 2001). In contrast, other results suggested that the peptide bond or structural peptide conformation can lead to both synergistic and antagonistic effects in comparison to the antioxidant activity of the amino acids on their own (Hernández-Ledesma et al., 2005). Eight constituent (P, S, K, Y, E, P, F and V) amino acids of the purified peptide (PSKYEPFV) from grass carp muscle hydrolysates mixed with the same concentration as the peptide did not show antioxidant activity (Ren et al., 2008). In addition, Zhang et al. (2010) also reported that six constituent amino acids (K, H, R, D, E and F) of the purified peptide (FRDEHKK), mixed with the same concentration as the peptide, did not show antioxidant activity. The authors concluded that amino acid sequence of peptide might play an important role on its activity. Elias et al. (2008) suggested that the higher antioxidant activity of peptides compared to free amino acids is attributed to the unique chemical and physical properties conferred by their amino acid sequences, especially the stability of the resultant peptide radicals that do not initiate or propagate further oxidative reaction.

2.4.3 Methods for antioxidant measurement

Specific assays have not yet been developed to measure antioxidant activity of peptides or peptide mixtures (Samaranayaka and Li-chan, 2011). Therefore, assays that are commonly employed to measure antioxidant activity of non-peptidic

antioxidants have been employed in the literature to measure the antioxidant activity of peptides (Samaranayaka and Li-chan, 2011). Due to the wide variety of oxidation processes and antioxidant action of protein hydrolysates or peptides, the use of a single method to evaluate the antioxidant activity cannot provide a clear idea about its real antioxidant potential (Ktari et al., 2012). Therefore, antioxidant activity could be determined using various assays.

2.4.3.1 *In vitro* chemical assays

There are several methods for determination of antioxidant activity of compounds, including inhibition of lipid oxidation, radicals scavenging and metal chelating ability. In term of foods, several methods of determining inhibition of lipid oxidation of food components include oxidation in fat; linoleic acid is usually used as a substrate to generate hydroperoxide because it is an important unsaturated fatty acid found in food (Kim, Kim, Kim, Oh, and Jung, 1994). Although this method is simple and rapid, it does not reflect the real food systems. Antioxidant activities using oxidation in food model system have been reported. Model meat system and pork patties are analyzed using the thiobarbituric acid (TBA) assay to detect thiobarbituric acid reactive substances (TBARS), such as malonaldehyde (MDA) (McCarthy, Kerry, Kerry, Lynch, and Buckley, 2001). However, this method is complex and time-consuming. Food generally consists of multiple phases in which lipids and water coexists with some emulsifier; therefore an antioxidant assay using a heterogeneous system, such as an emulsion is also required. β-Carotene bleaching method is widely used to measure the antioxidant activity of bioactive compounds because β-carotene is extremely susceptible to free radical mediated oxidation of linoleic acid (Sarkar, Bishayee, and Chatterjee, 1995).

Depending on the reactions involved, assays to assess antioxidant activity can be classified into two types: assays based on hydrogen atom transfer (HAT) and electron transfer (ET) reactions. The majority of HAT-based assays apply a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds, such as oxygen radical absorbance capacity (ORAC) (Cao, Alessio, and Cutler, 1993; Ou, Hampsch-Woodill, and Prior, 2001). The ORAC assay is considered to be more relevant because it utilizes a biologically relevant radical source. Although ORAC assay is sensitive, this assay requires detection by fluorometers, which may not be routinely available in analytical laboratories (Prior, Wu, and Schaich, 2005).

ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. The degree of color change is correlated with the antioxidant activity. ET-based assays include 2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Leong and Shui, 2002), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams, Cuvelier, and Berset, 1995), ferric reducing antioxidant power (FRAP) (Benzie and Strain, 1996). ABTS and DPPH assays are usually classified as ET reactions, these two indicator radicals in fact may be neutralized either by direction via electron transfers or by radical quenching via hydrogen atom transfer (Prior et al., 2005). FRAP assay measures reduction of ferric 2, 4, 6-tripyridyl-s-triazine (TPTZ) to a colored product, which is not relevant to antioxidant activity mechanistically. However, the reaction detects compounds with redox potentials of <0.7 V (the redox potential of Fe^{3+}-TPTZ), so, FRAP assay is a reasonable screen for the ability to maintain redox status in cells or tissue (Prior et al., 2005). Although these radicals (DPPH, ABTS and FRAP assays) do not reflect a

biologically relevant radical source and action in the biological system, these assays are usually used initially for screening antioxidative compounds because they are simple, inexpensive, and rapid. In addition, radical scavenging assays are helpful in evaluating the antioxidant mechanisms of a compound.

Furthermore, other assays intended to measure a sample's scavenging capacity of biologically relevant oxidants, such as superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$), are also reported. $O_2^{\bullet-}$ is known to be very harmful to cellular components as a precursor of more ROS, such as singlet oxygen ($^1O_2$) and $^{\bullet}OH$ (Aurand, Boonme, and Gidding, 1977). Therefore, study the scavenging effects of compound on $O_2^{\bullet-}$ is one of the most important ways of clarifying the mechanism of antioxidant activity. In this method, $O_2^{\bullet-}$ generated *in vitro* by the xanthine oxidase reduced the yellow dye (NBT^{2+}) to produce the blue formazan, which was measured spectrophotometrically at 560 nm. Among the oxygen radicals, the $^{\bullet}OH$ is the most reactive and severely damages adjacent biomolecules. Therefore, the removal of $^{\bullet}OH$ is probably one of the most effective defenses of a living body against various diseases. The scavenging effect against $HO^{\bullet}$ was investigated by using the 2-deoxyribose oxidation to MDA which reacts with TBA to the red compound, which was measured at 532 nm. Transition metals, such as $Fe^{2+}$and Cu^{2+}, are normally used to test metal chelating ability. In the Cu^{2+}chelation assay, the disappearance of the blue color, due to dissociation of Cu^{2+}, was monitored by determining the absorbance at 632 nm. In the Fe^{2+}chelation assay, the dark color of complex formed by the interaction of ferrozine with Fe^{2+} ions is decreased by the action of peptide that can be measured at 562 nm.

2.4.3.2 *In vitro* biological assays

Besides chemical assays, biological assays both *in vitro* and *in vivo* can

be performed. Although cellular model system studies may not reflect the different types of chemical and physical condition encountered by antioxidants *in vivo*, this type of assays has advantages in terms of cost, reproducibility, ability to control the environment, time, and good ethics (Raghavan et al., 2008). Cell culture experiment is a powerful technique for investigation of the normal physiology or biochemistry of cells and to test the effect of various chemical compounds or drugs on specific cell types. Cell culture models are widely used to evaluate cytotoxicity of antioxidative compounds at concentrations to be used to exert the desired bioactivity in the body.

The lactate dehydrogenase (LDH) leakage, Trypan blue (TB) and the 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assays are the most common employed for the detection of cytotoxicity or cell viability of toxic substances. Leakage of cytoplasmic enzymes into culture medium is a first parameter for a rapid and sensitive evaluation of cytotoxicity. Among cytosolic enzymes, LDH is one of the most commonly used cytotoxicity end-points. In addition, cell membrane damage leads to the release of cytoplasmic enzymes, and the measurement of LDH release is a well-accepted assay to estimate cell membrane integrity and quantify cell cytotoxicity (Ponsoda, Jover, Castell, and Gomez-Lechon, 1991). However, measurement of LDH leakage in the culture medium has some limitation. Not all LDH isoenzymes are stable in culture medium conditions, and the measured activity of leaked LDH may decrease during long term incubation, depending on the cell type (Ponsoda et al, 1991). For this reason, intracellular LDH after exposure of cells toxenobiotics was also measured for evaluating cytotoxicity (Gtmez-Lechtn, Ponsoda, and Jover, 1987). In this assay, homogenization of individual culture plates and determination of enzyme activity in homogenates are necessary.

This is not feasible in multiwell plates. Trypan blue dye exclusion assay is the most commonly utilized test for cell viability. The usefulness of this procedure is limited since the number of blue-staining cells increases following addition of the dye, requiring that cells be counted within 3-5 min (Altman, Randers, and Rao, 1993).

In *vitro* MTT assay is one of the most used methods for preliminary screening. It determines the ability of viable cells to convert a yellow water-soluble tetrazolium salt into insoluble purple formazan crystals by the mitochondrial dehydrogenase enzymes. The MTT assay is a rapid, versatile, quantitative, and highly reproducible colorimetric assay for mammalian cell viability (Liu and Zeng, 2009b). Cell culture experiment can also be used to study the potential of antioxidant compounds to inhibit intracellular oxidation.

2', 7'-dichlorofluorescin diacetate (DCFH-DA) assay is widely used to measure oxidative stress in cells due to the high sensitivity of this fluorescence-based assay (Bonini, Rota, Tomasi, and Mason, 2006). Intracellular oxidation of cells can be induced using a peroxy radical generator, hydrogen peroxide or using *tert*-butylhydroperoxide (TBHP) (Elisia, and Kitts, 2008; Qian et al., 2008a; Schantz, Mohn, Baum, and Richling 2010). DCFH-DA penetrates into the cells and is hydrolyzed to DCFH by intracellular esterases. The presence of ROS can oxidize DCFH to form 2', 7'-dichlorofluorescin (DCF), a fluorescent product. Evaluation of the intracellular ROS, such as H_2O_2, superoxide anion ($O^{\bullet-}_2$), singlet oxygen ($^1O_2$), and hydroxyl radicals ($^{\bullet}OH$) using fluorescent DCF can be used as an index of the overall oxidative stress within cells (Wang and Joseph, 1999).

The effects of compounds on cellular antioxidant enzyme levels can be measured using cell culture experiments. The antioxidant system is an important defense system

in living organisms because this system can eliminate reactive oxygen species (ROSs) (Yu, 1994). Liaw, Lee, Wu, Tsai, and Lin-Shiau (1997) suggested that low antioxidative enzyme levels in cancer cells may be due to the imbalanced redox status generated by excessive free radicals. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are the major defensive system against ROS (Sies, 1993). SOD detoxifies $O^{\bullet-}_2$, CAT reduces H_2O_2, while GPx reduces both H_2O_2 and organic peroxides.

2.4.3.3 *In vivo* assay

In vitro chemical antioxidant assays usually ignore biological actions *in vivo*, including the activity of antioxidant enzymes and oxidative related metabolism pathways, as well as the gene expression of antioxidant substances and enzymes (Liu, Kong, Xiong, and Xia, 2010). Once the antioxidative potential of a food constituent of interest is established using *in vitro* assay methods, *in vivo* assays, animal studies and human clinical trials can be conducted to confirm bioavailability and the desired biological function (Samaranayaka and Li-Chan, 2011). In addition, results from *in vivo* assays are an essential part in gaining approval from federal agencies for a dietary component to be used in functional food and nutraceutical formulations (Samaranayaka, and Li-Chan, 2011). Wang et al. (2008) reported that ingestion of Douchi (fermented soybean food) extracts of rats had an effect of increasing SOD activities in liver and kidney, CAT activity in liver, GPx activity in kidney as well as decreasing serum thiobarbituric acid reactive substances (TBARS) in liver and kidney. The authors suggested that peptides and free amino acids components of Douchi extracts act as antioxidant. In another *in vivo* study, following an intake of egg white hydrolysates (0.5 g/kg/day of egg white hydrolysates) by spontaneously

hypertensive rats (SHR), the radical-scavenging capacity of plasma rose while an elevated concentration of MDA in the aorta dropped (Manso et al., 2008). The authors postulated that egg white hydrolysates may contribute in prevention of oxidative stress by increment of plasma radical-scavenging capacity and inhibition of lipid peroxidation.

Sun, Pan, Guo, and Li (2012) assessed antioxidant activity of the fraction peptides of chicken breast protein hydrolysate *in vivo* using D-galactose-induced aging mice. When mice were treated with D-galactose, antioxidant enzyme (SOD, CAT and GPx activities decreased, and MDA levels increased as compared with the normal group (mice without treated D-galactose) both in serums and livers. D-galactose-induced aging mice administrated the fraction peptides of chicken breast protein hydrolysate (125, 250 and 500 mg/kg) showed significantly increased antioxidant enzyme activities, while MDA levels decreased both in serums and livers. However, no statistical significant difference between the three peptide doses was observed. In addition, the authors suggested that under a transmission electron microscope (TEM), the ultramicrostructure of hepatic tissue was observed and the hydrolysate may play a part in inhibiting oxidative stress in hepatocytes *in vivo*. You, Zhao, Regenstein, and Ren (2011) also reported that Loach (*Misgurnus anguillicaudatus*) peptides improved the endogenous cellular antioxidant enzymes in mice by increasing the activities of SOD, CAT and GPx.

2.4.4 Antioxidant peptide in cell model systems and their mechanisms

Several studies used cell culture to assess the protective effect of peptide on radical-mediated cellular injuries and death. TBHP and H_2O_2 have been widely used as an inducer of oxidative stress to mimic oxidative stress induced injury (Aherne and

O'Brien, 2000; Alía, Ramos, Mateos, Bravo, and Goya, 2005). It is well known that TBHP rapidly penetrates into mammalian cells. The proposed mechanisms of TBHP-induced toxicity is related to decomposition of TBHP to reactive alkoxyl and peroxyl radicals catalyzed by cytochrome P450, initiating peroxidation of membrane lipids (Lapshina, Zavodnik, Labieniec, Rekawiecka, and Bryszewska, 2005). Peroxidation of membrane lipids affects cell integrity and forms covalent bonds with cellular molecules resulting in TBHP-induced toxicity and cell death. In addition, decomposition of TBHP can induce cell toxicity by damage to DNA and is associated with the loss of mitochondrial function (Aherne and O'Brien, 2000). This decomposition is aided by metal ions and their complexes, leading to the generation of different ROS, including H_2O_2 (Coleman, Gilfor, and Farber, 1989; Guidarelli, Cattabeni, and Cantoni, 1997). It is well known that H_2O_2 itself is not highly reactive; however, it forms highly reactive oxygen species ($^{\bullet}OH$) in the presence of transition metalions and through other mechanisms (Halliwell and Gutteridge, 1992). The formation of $^{\bullet}OH$ and other ROS initiates lipid peroxidation and causes DNA damage (Zhang, Wang, Xu, and Gao, 2009).

Park, Jung, Nam, Shahidi, and Kim (2001) reported that peptide from lecithin-free egg yolk; LELHKLRSSHWFSRR can protect normal human liver cells from lipid peroxidation induced by TBHP at low peptide concentration of 10 µg/ml, as determined by the accumulation of MDA, as measured by TBARS in the medium. Kim et al. (2001) reported that purified peptide, GPOGPOGPOGPOG, from Alaska pollack skin can enhanced cell viability of Donryu rat liver cells from oxidant injury by TBHP at 1 mg/ml dosage, as evaluated by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide MTT assay. Two purified peptides, NADFGLNGLEGLA

(1307 Da) and NGLEGLK (747 Da) from giant squid muscle enhanced the viability of cytotoxic embryonic lung fibroblasts MRC-5 cells induced by TBHP at a low concentration of 50µg/ml, and it was presumed due to the suppression of radical-induced oxidation of membrane lipids (Rajapakse et al., 2005a). Furthermore, the authors also suggested that the higher activity of NGLEGLK can be attributed to the lower molecular weight that improves contact ability with membrane lipids and/or permeability.

Two purified peptides, FDSGPAGVL (880.18 Da) and NGPLQAGQPGER (1241.59 Da), from jumbo squid skin gelatin also enhanced cell viability of human lung fibroblasts MRC-5 cells induced by TBHP oxidation in a dose-dependent manner ranging from 25-100 µg/ml (Mendis et al., 2005a). However, cell viability enhancement effect of these two peptides was comparable. The authors suggested that molecular weight and amino acid sequence do not affect on cytoprotective effect of these two peptides. They hypothesized that hydrophobic nature of peptides facilitated scavenging of free radicals in cellular system by keeping close contact with oxidizing cell membrane lipids.

Kim et al. (2007) also reported that purified peptide ESTVPERTHPACPDFN (1801 Da) from hoki frame can overcome TBHP-induced cytotoxicity on human embryonic lung fibroblasts MRC-5 cells by scavenging lipid-derived radicals in dose-dependent increments of 2.78-55.5µM. In addition, the authors also suggested that this purified peptide can protect hydroxyl radical derived from a Fenton reaction - induced plasmid pBR 322 DNA damage in *vitro*.

Qian, et al. (2008a) reported that purified peptide from oyster, LKQELEDLLEKQE (1600 Da) showed no cytotoxicity on human embryonic lung

fibroblasts MRC-5 and the mouse macrophages cell line (RAW264.7), as evaluated by MTT assay. In addition, purified peptide from oyster can also scavenge cellular radicals -induced by H_2O_2 in the mouse macrophages cell line (RAW264.7) in a dose-dependent manner (10-100 µg/ml), as determination by non-toxic fluorescence dye, DCFH-DA. Furthermore, peptide can protect the DNA damage caused by generating hydroxyl radicals at concentration of 5.7 to 45.6 µM.

Je et al. (2008) reported that the purified peptide isolated from the bigeye tuna, LNLPTAVYMVT (1222 Da), can inhibit radical-induced by H_2O_2 production in human fibrosarcoma cells (HT1080) at a concentration of 50 µg/ml. The authors suggested that the potent radical scavenging effects were due to the presence of Pro, Leu, Ala, and Tyr. In addition, this peptide can protect cell from radical-mediated cellular injuries by TBHP and viability of the human embryonic lung fibroblasts MRC-5 cell line increased with increasing concentrations (5.48-43.8 µM).

Synthetic FRDEHKK (959.5 Da) from rice endosperm significantly enhanced the viability of TBHP induced cytotoxicity up to 74.38% (for the human embryonic lung fibroblasts) and 78.39% (for the mouse macrophage) at a concentration of 80 µg/ml, as measured by MTT assay (Zhang et al., 2010). In addition, this peptide can scavenge intracellular ROS induced by H_2O_2 in mouse macrophages, RAW 264.7 cells in a dose-dependent manner of 10-100 µg/ml.

MTT assay and lactate dehydrogenase (LDH) assay have been used to evaluate the protection of peptides from water buffalo horn in rat cerebral microvascular endothelial cells (rCMECs) against H_2O_2-induced injury (Liu et al., 2010). They found that three purified peptides, Q**Y**DQ**G**V (708 Da), YED**C**TD**C**GN (1018 Da) and AADNANEL**FPP**N (1271 Da), protected rCMECs cell against H_2O_2-

induced injury. In addition, those peptides could significantly reduce apoptotic cells, indicating that peptides protected rCMECs against H_2O_2-induced apoptosis. The authors suggested that the proton-donator amino acids, Tyr, Gly, Cys and Phe-Pro-Pro (FPP) residues may play an important role in scavenging radicals through direct electron transfer. Furthermore, hydrophobic amino acids, such as Gly, Ala, Val, Leu, Trp, Pro and Phe, might increase an affinity and reactivity towards the cell membrane in the rCMECs, leading to a better antioxidant efficacy.

Sheih et al. (2009a) reported that human gastric cancer cell lines (AGS) pre-incubated with various concentrations of purified antioxidative peptides derived from micro algae had a dose-dependent protective effect from H_2O_2-induced oxidative damage. Furthermore, they also reported that no *in vitro* cytotoxicity of purified peptide in human lung fibroblasts cell lines (WI-38) was observed. Some of the common bean hydrolysates have antioxidant activity in Caco-2 cell cultures treated with the free radical generator 2, 2'-azobis (2-amidinopropane) dihydrochloride (ABAP) (Carrasco-Castilla et al., 2012). Mendis et al. (2005b) reported that purified gelatin hydrolysate from hoki skin containing amino acid sequence of HGPLGPL (797 Da) increased the level of Hep 3B cellular antioxidative enzymes (SOD, GPx and CAT). However, none of these studies have elucidated a mechanism of antioxidants in promoting natural cellular antioxidant system. They suggested that the peptide may be involved in maintaining the redox balance because of its radical-scavenging properties. Phelan, Aherne-Bruce, O'Sullivan, FitzGerald, and O'Brien (2009) reported human Jurkat T cells, whereas none of the samples significantly affected SOD activity. In addition, the authors suggested that when casein hydrolysate was added to the cells at concentrations ≤1% (v/v), none of the casein hydrolysates

affected cell membrane integrity as evaluated by LDH release assay.

Kong et al. (2012) reported that purified peptide from whey protein hydrolysate, VHLKP had efficacy against hydrogen peroxide (H_2O_2)-induced human lung fibroblast MRC-5 cell oxidative injury that can be attributed to peptides' ability to neutralize radicals and other reactive oxygen species and promoting the activity of intracellular antioxidative enzymes (SOD, CAT, and GPx). The authors also suggested that the small peptide size and the presence of two strongly hydrophobic amino acid residues (Val and Leu) in this peptide would allow it to be absorbed and transported through lipid membrane and release its bioactivity in the MRC-5 cell.

Himaya, Ngo, Ryu, and Kim. (2012) reported that the purified peptide from Pacific cod (*Gadus macrocephalus*) skin hydrolysate, LLMLDNDLPP (1301 Da) significantly reduced the oxidation levels of membrane lipids, proteins and DNA in RAW264.7 cells by effectively scavenging the intracellular ROS. The authors suggested that having low molecular weight (1301 Da), 70% hydrophobicity and presence of two Leu residues at the N terminal could contribute antioxidant activity. Increased hydrophobicity of the peptide enhances the antioxidant activity of a peptide, as it allows the peptide to reach hydrophobic targets like cell membranes (Hsu, 2010). Moreover, this LLMLDNDLPP also up regulated the m-RNA expression of cellular antioxidative enzymes (SOD, GPx and CAT) and thereby enhanced the intracellular antioxidant mechanisms.

2.4.5 Potential application of antioxidant

Protein hydrolysates and peptides have potential application as functional ingredients in food systems to reduce oxidative changes during storage. Protein hydrolysates derived from elastin were effective inhibitors of the oxidation of oleic

acid (Hattori et al., 1998). In addition, it has been reported that protein hydrolysate derived from egg york effectively inhibited thiobarbituric acid reactive substances (TBARS) formation from ground beef and tuna homogenates (Sakanaka, and Tachibana, 2006). Fitzgerald (1998) also reported that antioxidative caseinophosphopeptides prepared by tryptic digestion of casein, have been used in breakfast cereals, breads, pastry, chocolate, juices, tea, and mayonnaise. However, stability of antioxidant peptides during food processing and storage should be assessed before commercializing a particular protein hydrolysate of interest for incorporation into a food product (Samaranayaka and Li-Chan, 2011).

Until now, most of the biological effects of protein hydrolysates and peptides have been assessed *in vitro* or in animal models. Only a few commercial products from protein hydrolysates with antioxidant properties are available to date. This may be attributed to several reasons, including a lack of clinical trials (to confirm bioactivity, efficacy, and safety, high production cost, problems in making a reproducible product, bitterness, color, or other organoleptic problems (Samaranayaka and Li-Chan, 2011).

For instance, fish protein hydrolysates are already sold as food supplements in Europe and North America markets, such as Fortidium Liquamen®, and Seacure®. Fortidium Liquamen®, a white fish (*Molva molva*) autolysate is commercialized as antioxidant, lowers glycemic index and anti-stress (Guerard et al., 2010). Seacure® is a commercialized fermented Pacific whiting or hake (*Merluccius productus*) with a proteolytic *Hansenula* yeast culture (Englender, 2000). Seacure® is clamed to reduce the degree of small intestine damage caused by non-steroid anti-inflammatory drugs in a pilot human clinical trial (Marchbank, Limdi, Mahmood, Elia, and Playford,

2008). The authors also suggested that glutamine present in fish protein hydrolysate might have contributed to antioxidant activity via stimulation of glutathione production.

2.5 Reference

Aherne, S. A. and O'Brien, N. M. (2000). Mechanism of protection by the flavonoids, quercetin and rutin, against tert-butylhydroperoxide and menadione-induced DNA single strand breaks in Caco-2 cells. **Free Radical Biology and Medicine.** 29: 507-514.

Aleman, A., Gimenez, B., Montero, P., and Gomez-Guillen, M. C. (2011). Antioxidant activity of several marine skin gelatins. **Lwt-Food Science and Technology.** 44: 407-413.

Alía, M., Ramos, S., Mateos, R., Bravo, L., and Goya, L. (2005). Response of the antioxidant defense system to t-Butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). **Journal of Biochemical and Molecular Toxicology.** 19: 119-128.

Altman, S. A., Randers, L., and Rao, G. (1993). Comparison of Trypan Blue Dye Exclusion and Fluorometric Assays for Mammalian Cell Viability Determinations. **Biotechnology Progress.** 9: 671-674.

Aurand, L. W., Boonme, N. H., and Gidding, G. G. (1977). Superoxide and singlet oxygen in milk lipid peroxidation. **Journal of Dairy Science**. 60: 363-369.

Authen, R. L., Davis, J. M. (2009). Oxygen toxicity and reactive oxygen species: the devil is in the details. **Pediatric Research**. 66: 121-127.

Balaban, R. S., Nemoto, S., and Finkel, T. (2005). Mitochondria, oxidants, and aging.

Cell. 120: 483-495.

Bamdad, F., Wu, J., and Chen, L. (2011). Effects of enzymatic hydrolysis on molecular structure and antioxidant activity of barley hordein. **Journal of Cereal Science.** 54: 20-28.

Batista, I., Ramos, C., Coutinho, J., Bandarra, N. M., and Nunes, M. L. (2010). Characterization of protein hydrolysates and lipids obtained from black scabbardfish (*Aphanopus carbo*) by-products and antioxidative activity of the hydrolysates produced. **Process Biochemistry.** 45: 18-24.

Benjakul, S. and Morrissey, M. T. (1997). Protein hydrolysates from Pacific whiting solid wastes. **Journal of Agricultural and Food Chemistry**. 45: 3423-3430.

Benjakul, S., Chantarasuwan, C., and Visessanguan, W. (2003). Effect of medium temperature setting on gelling characteristics of surimi from some tropical fish. **Food Chemistry**. 82: 567-574.

Benzie, I. F. F. and Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. **Analytical Biochemistry.** 239: 70-76.

Bielski, B. H. J. and Cabelli, D. E. (1991). Highlights of current research involving superoxide and perhydroxyl radicals inaqueous solutions. **The International Journal of Radiation Biology.** 59: 292-319.

Bonini, M. G., Rota, C., Tomasi, A., and Mason, R. P. (2006). The oxidation of 2', 7'-dichlorofluorescin to reactive oxygen species: a self-fulfilling prophesy? **Free Radical Biology and Medicine.** 40: 968-75.

Bougatef, A., Hajji, M., Balti, R., Lassoued, I., Triki-Ellouz, Y., and Nasri, M. (2009). Antioxidant and free radical-scavenging activities of smooth hound (*Mustelus*

mustelus) muscle protein hydrolysates obtained by gastrointestinal proteases. **Food Chemistry.** 114: 1198-1205.

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

Bourtoom, T., Chinnan, M. S., Jantawat, P., and Sanguandeekul, R. (2009). Recovery and characterization of proteins precipitated from surimi wash-water. **LWT-Food Science and Technology**. 42: 599-605.

Brand-Williams, W., Cuvelier, M. E., and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. **Lebensmittel Wissenschaft and Technologie**. 28: 25-30.

Brown, W. E., and Wold, F. (1973). Alkyl isocyanates as active-site-specific reagents for serine proteases. Identification of the active-site serine as the site of reaction. **Biochemistry.** 12: 835-840.

Buonocore, G., Perrone, S., and Tataranno, M. L. (2010). Oxygen toxicity: chemistry and biology of reactive oxygen species. **Seminars in Fetal and Neonatal Medicine.** 15: 186-190.

Byun, H. G., Lee, J. K., Park, H. G., Jeon, J. K., and Kim, S. K. (2009). Antioxidant peptides isolated from the marine rotifer, *Brachionus rotundiformis*. **Process Biochemistry.** 44: 842-846.

Cadenas, E. (1989). Biochemistry of oxygen toxicity. **Annual Review of Biochemistry.** 58: 79-110.

Cao, G., Alessio, H. M., and Cutler, R. G. (1993). Oxygen-radical absorbance capacity

assay for antioxidants. **Free Radical Biology and Medicine.** 14: 303-311.

Carrasco-Castilla, J., Hernández-Álvarez, A. J., Jiménez-Martínez, C., Jacinto-Hernández, C., Alaiz, M., Girón-Calle, Vioque, J., and Dávila-Ortiz, G. (2012). Antioxidant and metal chelating activities of peptide fractions from phaseolin and bean protein hydrolysates. **Food Chemistry** 135: 1789-1795.

Chang, C. Y., Wu, K. C., and Chiang, S. H. (2007). Antioxidant properties and protein compositions of porcine haemoglobin hydrolysates. **Food Chemistry**. 100: 1537-1543.

Chen, H. M., Muramoto, K., and Yamauchi, F. (1995). Structural analysis of antioxidative peptides from soybean β-Conglycinin. **Journal of the Science of Food and Agriculture**. 43: 574-578.

Chen, H. M., Muramoto, K., Yamauchi, F., Fujimoto, K., and 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

Chen, H. M., Muramoto, K., Yamauchi, F., and Nokihara, K. (1996). Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean peptide. **Journal of Agricultural and Food Chemistry**. 44: 2619-2623.

Chen, N., Yang, H., Sun, Y., Niu, J., and Liu, S. (2012). Purification and identification of antioxidant peptides from walnut (*Juglans regia* L.) protein hydrolysates. **Peptides**. 38: 344-349.

Cheung, I. W. Y., Cheung, L. K. Y., Tan, N. Y., and Li-Chan, E. C. Y. (2012). The role of molecular size in antioxidant activity of peptide fractions from Pacific

hake (Merluccius productus) hydrolysates. **Food Chemistry.** 134: 1297-1306.

Chow, C. K. (1988). Interrelationships of cellular antioxidant defense systems. In C. K. Chow (Ed.). In **Cellular Antioxidant Defense Mechanisms** (pp. 217-237). Boca Raton, FL: CRC Press.

Coleman, J., Gilfor, D., and Farber, J. L. (1989). Dissociation of the accumulation of single-strand breaks in DNA from the killing of cultured hepatocytes by an oxidative stress. **Molecular Pharmacology.** 36: 193-200.

Conner, E. M. and Grisham, M. B. (1996). Inflammation, free radicals, and antioxidants. **Nutrition.** 12: 274-277.

Cornelli, U. (2009). Antioxidant use in nutraceuticals. **Clinics in Dermatology.** 27: 175-194.

Cumby, N., Zhong, Y., Naczk, M., and Shahidi, F. (2008). Antioxidant activity and water-holding capacity of canola protein hydrolysates. **Food Chemistry.** 109: 144-148.

Di Bernardini, R., Rai, D. K., Bolton, D., Kerry, J., O'Neill, E., Mullen, A. M., Harnedy, P., and Hayes, M. (2011). Isolation, purification and characterization of antioxidant peptidic fractions from a bovine liver sarcoplasmic protein thermolysin hydrolyzate. **Peptides.** 32: 388-400.

Dong, S., Zeng, M., Wang, D., Liu, Z., Zhao, Y., and Yang, H. (2008). Antioxidant and biochemical properties of protein hydrolysates prepared from Silver carp (*Hypophthalmichthys molitrix*). **Food Chemistry.** 107: 1485-1493.

Doucet, D., Otter, D. E., Gauthier, S. F., and Foegeding, E. A. (2003). Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: Peptide identification and determination of enzyme specificity. **Journal of Agricultural**

and Food Chemistry. 50: 6300-6308.

Elias, R. J., Kellerby, S. S., and Decker, E. A. (2008). Antioxidant activity of proteins and peptides. **Critical Reviews in Food Science and Nutrition.** 48: 430-441.

Elisia, I. and Kitts, D. D. (2008). Anthocyanins inhibit peroxy radicalinduced apoptosis in Caco-2 cells. **Molecular and Cellular Biochemistry.** 312: 139-145.

Englender, C. (2000). Symptom reduction in irritable bowel syndrome with pre-digested fish protein supplement. **Townsend Letter.** 205/206: 60-64.

Finkel, T. (2005). Radical medicine: treating ageing to cure disease**. Nature Reviews Molecular Cell Biology**. 6: 971-976.

Fitzgerald, R. J. (1998). Potential uses of caseinophosphopeptides. **International Dairy Journal.** 8: 451-457.

Folk, J. E. and Schirmer, E. W. (1965). Chymotrypsin. Isolation of zymogen and active enzyme-preliminary structure and specificity studies. **Journal of Biological Chemistry**. 240: 181-189.

Fridovich, I. (1995). Superoxide radical and superoxide dismutases. **Annual Review of Biochemistry**. 64: 97-112.

Garnjanagoonchorn, W., Nitisinprasert, S., and Yamprai.S. (1998). The utilization of by-product from surimi industry: laboratory scale production of spray dried fish protein hydrolysate. **Kasetsart Journal: Natural Science.** 32: 28-40.

Gtmez-Lechtn, M. J., Ponsoda, X., and Jover, R. (1987). Hepatotoxicity of the opioids morphine, heroin, meperidine and methadone to cultured human hepatocytes. **Molecular and Cellular Toxicology.** 1: 453-463.

Gómez-Ruiz, J. Á., López-Expósito, I., Pihlanto, A., Ramos, M., and Recio, I. (2008). Antioxidant activity of bovine casein hydrolysates: identification of active

peptides by HPLC–MS/MS. **European Food Research and Technology.** 227: 1061-1067.

Grice, H. C. (1986). Safety evaluation of butylated hydroxytoluene (BHT) in the liver, lung and gastrointestinal tract. **Food and Chemical Toxicology.** 24: 1127-1130.

Gu, L., Zhao, M., Li, W., You, L., Wang, J., Wang, H., and Ren, J. (2012). Chemical and cellular antioxidant activity of two novel peptides designed based on glutathione structure. **Food and Chemical Toxicology.** 50: 4085-4091.

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

Guidarelli, A., Cattabeni, F., and Cantoni, A. (1997). Alternative mechanisms for hydroperoxide-induced DNA single strand breaks. **Free Radical Research.** 26: 537-547.

Guo, H., Kouzuma, Y., and Yonekura, M., (2009). Structures and properties of antioxidative peptides derived from royal jelly protein. **Food Chemistry**.113: 238-245.

Halliwell, B. (1996). Antioxidants in human health and disease. **Annual Review of Nutrition.** 16: 33-50.

Halliwell, B. (2007). Biochemistry of oxidative stress. **Biochemical Society Transactions**. 35: 1147-1150.

Halliwell, B. and Gutteridge, J. M. C. (1990). Role of free radicals and catalytic metal ions in human disease: an overview. **Methods in Enzymology**. 186: 1-85.

Halliwell, B. and Gutteridge, J. M. C. (1992). Biologically relevant metal ion-depend-dent hydroxyl radical generation.Anupdate. **FEBS Letters.** 307: 108-112.

Halliwell, B. and Gutteridge, J. M. C. (1999). **Free Radicals in Biology and Medicine** (3rd ed.). Oxford University Press.

Hardy, J. A. and Higgins, G. A. (1992). Alzheimer's disease: The amyloid cascade hypothesis. **Science.** 256: 184-185.

Hattori, M., Yamaji-Tsukamoto, K., Kumagai, H., Feng, Y., and Takahashi, K. (1998). Antioxidative Activity of Soluble Elastin Peptides. **Journal of Agricultural and Food Chemistry.** 46: 2167-2170.

Hernández-Ledesma, B., Davalos, A., Bartolome, B., and Amigo, L. (2005). Preparation of antioxidant ensymatichydrolysates from α-lactalbumin and β-lactoglobulin.Identification of active peptides by HPLC-MS/MS. **Journal of Agricultural and Food Chemistry.** 53: 588-593.

Himaya, S. W. A., Ngo, D. H., Ryu, B., and Kim, S. K. (2012). An active peptide purified from gastrointestinal enzyme hydrolysate of Pacific cod skin gelatin attenuates angiotensin-1 converting enzyme (ACE) activity and cellular oxidative stress. **Food Chemistry.** 132: 1872-1882.

Hogan, S., Zhang, L., Li, J., Wang, H., and Zhou, K. (2009). Development of antioxidant rich peptides from milk protein by microbial proteases and analysis of their effects on lipid peroxidation in cooked beef. **Food Chemistry.** 117: 438-443.

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

Huang, D., Boxin, O. U., and Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. **Journal of Agricultural and Food Chemistry.** 53: 1841-1856.

Hwang, J. Y., Shyu, Y. S., Wang, Y. T., and Hsu, C. K. (2010). Antioxidative properties of protein hydrolysate from defatted peanut kernels treated with esperase. **LWT - Food Science and Technology.** 43: 285-290.

Inoue, M., Sato, E. F., and Nishikawa M. (2003). Mitochondrial generation of reactive oxygen species and its role in aerobic life. **Current Medicinal Chemistry.** 10: 2495-2505.

Jamdar, S. N., Rajalakshmi, V., Pednekar, M. D., Juan, F., Yardi, V., and Sharma, A. (2010). Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. **Food Chemistry.** 121: 178-184.

Jamilah, B. and Harvinder, K. G. (2002). Properties of gelatins from skins of fish-black tilapia (*Oreochromis massambicus*) and red tilapia (*Oreochromis nilotica*). **Food Chemistry.** 77: 81-84.

Je, J. Y., Lee, K. H., Lee, M. H., and Ahn, C. B. (2009). Antioxidant and antihypertensive protein hydrolysates produced from tuna liver by enzymatic hydrolysis. **Food Research International.** 42: 1266-1272.

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

Je, J. Y., Qian, Z. J., Lee, S. H., Byun, H. G., and Kim, S. K. (2008). Purification and antioxidant properties of Bigeye Tuna (*Thunnus obesus*) dark Muscle peptide

on free Radical-mediated oxidative systems. **Journal of Medicinal Food.** 11: 629-637.

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

Jongjareonrak, A., Benjakul, S., Visessanguan, W., and Tanaka, M., (2005). Isolation and characterization of collagen from bigeye snapper (*Priacanthus macracanthus*) skin. **Journal of the Science of Food and Agriculture.** 85: 1203-1210.

Jornot, L., Petersen, H., and Junod, A. F. (1998). Hydrogen per-oxide-induced DNA damage is independent of nuclear calcium but dependent on redox-active ions. **Biochemical Journal.** 335: 85-94.

Jun, S. Y., Park, P. J., Jung, W. K., and 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., Rajapakse, N., and Kim, S. K. (2005). Antioxidative activity of a low molecular weight peptide derived from the sauce of fermented blue mussel, *Mytilus edulis.* **European Food Research and Technology.** 220: 535-539.

Kanjanapongkul, K., Yoovidhya, T., Tia, S., and Wongsa-Ngasri, P. (2008). Protein removal from fish mince washwater using ohmic heating. **Songklanakarin Journal of Science and Technology.** 30: 413-419.

Kawanishi, S., Hiraku, Y., Murata., M, and Oikawa, S. (2002). The role of metals in site-specific DNA damage with reference to carcinogenesis. **Free Radical**

Biology and Medicine. 32: 822-32.

Kawashima, K., Itoh, H., Miyoshi, M., and Chibata, I. (1979). Antioxidant properties of branched-chain amino acid derivatives. **Chemical and Pharmaceutical Bulletin.** 27: 1912-1916.

Khantaphant, S. and 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.** 151: 410-419.

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

Kim, S. K., Kim, Y. T., Byun, H. G., Nam, K. S., Joo, D. S., and 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., Kim, J. H., Kim, S. K., Oh, M. J., and Jung, M. Y. (1994). Antioxidant activities of selected oriental herb extracts. **Journal of the American Oil Chemists' Society.** 71: 633-640.

Kim, E. K., Lee, S. J., Jeon, B. T., Moon, S. H., Kim, B., Park, T. K., Han, J. S., and Park, P. J. (2009). Purification and characterisation of antioxidative peptides from enzymatic hydrolysates of venison protein. **Food Chemistry.** 114: 1365-1370.

Kim, J. S. and Park, J. W. (2005). Partially purified collagen from refiner discharge of Pacific Whiting surimi processing. **Journal of Food Science**. 70: c511-c516.

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

Klaunig, J. E., Xu, Y., Bachowski, S., and Jiang, J. (1997). Free-radical oxygen-induced changes in chemical carcinogenesis. In K. B. Wallace (ed.). **Free radical toxicology.** (pp. 375-400). London: Taylor & Francis.

Klompong, V., Benjakul, S., Kantachote, D., Hayes, K. D., and Shahidi, F. (2008). Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from Alcalase and Flavourzyme. **International Journal of Food Science and Technology.** 43: 1019-1026.

Ko, J. Y., Lee, J. H., Samarakoon, K., Kim, J. S., and Jeon, Y. J. (2013). Purification and determination of two novel antioxidant peptides from flounder fish (*Paralichthys olivaceus*) using digestive proteases. **Food and Chemical Toxicology.** 52: 113-120.

Kojo, S. (2004). Vitamin C: basic metabolism and its function as an index of oxidative stress. **Current Medicinal Chemistry**. 11: 1041-1064.

Kong, B., Peng, X., Xiong, Y. L., and Zhao, X. (2012). Protection of lung fibroblast MRC-5 cells against hydrogen peroxide-induced oxidative damage by 0.1-2.8Da antioxidative peptides isolated from whey protein hydrolysate. **Food Chemistry.** 135: 540-547.

Kong, B. H. and Xiong, Y. L. L. (2006). Antioxidant activity of zein hydrolysates in a liposome system and the possible mode of action. **Journal of Agricultural and Food Chemistry.** 54: 6059-6068.

Kou, X., Gao, J., Xue, Z., Zhang, Z., Wang, H., and Wang, X. (2013). Purification and

identification of antioxidant peptides from chickpea (*Cicer arietinum* L.) albumin hydrolysates. **LWT - Food Science and Technology.** 50: 591-598.

Ktari, N., Jridi, M., Bkhairia, I., Sayari, N., Ben Salah, R., and Nasri, M. (2012). Functionalities and antioxidant properties of protein hydrolysates from muscle of zebra blenny (*Salaria basilisca*) obtained with different crude protease extracts. **Food Research International.** 49: 747-756.

Kudo, K., Onodera, S., Takeda, Y. Benkeblia, N., and Shiomi, N. (2009). Antioxidative activities of some peptides isolated from hydrolyzed potato protein extract. **Journal of Functional Foods.** 1: 170-176.

Lahl, W. J. and Braun, S. D. (1994). Enzymatic production of protein by hydrolysates for food use. **Food Technology.** 48: 68-71.

Lapshina, E. A., Zavodnik, I. B., Labieniec, M., Rekawiecka, K., and Bryszewska, M. (2005). Cytotoxic and genotoxic effects of *tert*-butyl hydroperoxide on Chinese hamster B14 cells. **Mutation Research**. 583: 189-97.

Leonarduzzi, G., Sottero, B., and Poli, G. (2010). Targeting tissue oxidative damage by means of cell signaling modulators: the antioxidant concept revisited. **Pharmacology and Therapeutics.** 128: 336-74.

Leong, L. P. and Shui, G. (2002). An investigation of antioxidant capacity of fruits in Singapore markets. **Food Chemistry.** 76: 69-75.

Li, Y., Jiang, B., Zhang, T., Mu, W., and Liu, J. (2008). Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). **Food Chemistry.** 106: 444-450.

Liaw, K. Y., Lee, P. H., Wu, F. C., Tsai, J. S., and Lin-Shiau, S. Y. (1997). Zinc, copper, and superoxide dismutase in hepatocellular carcinoma. **American**

Journal of Gastroenterology. 92: 2260-2263.

Lin, L. and Li, B. F. (2006). Radical scavenging properties of protein hydrolysates from jumbo flying squid (*Dosidicus eschrichitii Steenstrup*) skin gelatin. **Journal of the Science of Food and Agriculture.** 86: 2290-2295.

Liochev, S. I. and Fridovich, I. (1994). The role of O_2 in the production of HO: *In vitro* and *in vivo*. **Free Radical Biology and Medicine.** 16: 29-33.

Liochev, S. I. and Fridovich, I. (2002). The Haber–Weiss cycle—70 years later: An alternative view**. Redox Report.** 7: 55-57.

Liu, Z., Dong, S., Xu, J., Zeng, M., Song, H., and Zhao, Y. (2008). Production of cysteine-rich antimicrobial peptide by digestion of oyster (*Crassostrea gigas*) with alcalase and bromelin. **Food Control.** 19: 231-235.

Liu, Q., Kong, B., Jiang, L., Cui, X., and Liu, J. (2009a). Free radical scavenging activity of porcine plasma protein hydrolysates determined by electron spin resonance spectrometer. **LWT - Food Science and Technology.** 42: 956-962.

Liu, Q., Kong, B., Xiong, Y. L., and Xia, X. (2010). Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. **Food Chemistry.** 118: 403-410.

Liu, J. H., Tian, Y. G., Wang, Y., Nie, S. P., Xie, M. Y., Zhu, S., Wang, C. Y., and Zhang, P (2011). Characterization and in vitro antioxidation of papain hydrolysate from black-bone silky fowl (*Gallus gallus domesticus Brisson*) muscle and its fractions. **Food Research International.** 44: 133-138.

Liu, Z. H. and Zeng., S. (2009b). Cytotoxicity of ginkgolic acid in HepG2 cells and primary rat hepatocytes. **Toxicology Letters.** 187: 131-136.

Loguercio, C. and Federico, A. (2003). Oxidative stress in viral and alcoholic Hepatitis.

Free Radical Biology and Medicine. 34: 1-10.

Manso, M. A., Miguel, M., Even, J., Hernaĭndez, R., Aleixandre, A., and Loĭpez-Fandin˜o, R. (2008). Effect of the long-term intake of an egg white hydrolysate on the oxidative status and blood lipid profile of spontaneously hypertensive rats. **Food Chemistry**. 109: 361-367.

Marchbank, T., Limdi, J. K., Mahmood, A., Elia, G., and Playford, R. J. (2008). Clinical trial: Protective effect of a commercial fish protein hydrolysate against indomethacin (NSAID)-induced small intestinal injury. **Alimentary Pharmacology and Therapeutics.** 28: 799-804.

Matés, J. M., Perez-Gomez, C., and Núñez de Castro, I. (1999). Antioxidant enzymes and human diseases. **Clinical Biochemistry**. 32: 595-603

Matés, J. M. and Sánchez-Jiménez, F. (1999). Antioxidant enzymes and their implications in pathophysiologic processes. **Frontiers in Bioscience**. 4: 339-345.

McCarthy, T. L., Kerry, J. P., Kerry, J. F, Lynch, P. B., and Buckley, D. J. (2001). Assesment of the antioxidant potential of natural food and plant extracts in fresh and previous frozen pork patties. **Meat Science**. 57: 177-184.

Meisel, H. and FitzGerald, R. J. (2003). Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. **Current Pharmaceutical Design.** 9: 1289-95.

Memarpoor-Yazdi M., Asoodeh A., Chamani J. (2012). A novel antioxidant and antimicrobial peptide from hen egg white lysozyme hydrolysates. **The Journal of Functional Foods.** 4: 278-286.

Memarpoor-Yazdi, M., Mahaki, H., and Zare-Zardini, H. (2013). Antioxidant activity of protein hydrolysates and purified peptides from Zizyphus jujuba fruits.

Journal of Functional Foods. 5: 62-70.

Mendis, E., Rajapakse, N., Byun, H. G., and Kim, S. K. (2005a). Investigation of jumbo squid (*Dosidicus gigas*) skin gelatin peptides for their *in vitro* antioxidant effects. **Life Sciences.** 77: 2166-2178.

Mendis, E., Rajapakse, N., and Kim, S. K. (2005b). Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. **Journal of Agricultural and Food Chemistry.** 53: 581-587.

Mielnik, M. B., Aaby, K., and Skrede, G. (2003). Commercial antioxidants control lipid oxidation in mechanically deboned turkey meat. **Meat Science.** 65: 1147-1155.

Miller, D. M., Buettner, G. R., and Aust, S. D. (1990).Transition metals as catalysts of "autoxidation" reactions. **Free Radical Biology and Medicine.** 8: 95-108.

Mine, Y. and Katayama, S. (2008). Antioxidative Stress Peptides. **Functional Food and Health.** 993: 213-228.

Morrisey, M. T., Lin, J., and Ismond, A. (2005). Waste management and waste utilization. In J. W. Park (Ed.). **Surimi and surimi seafood** (second ed., pp. 279-323). New York: Marcel Dekker.

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

Mullally, M. M., OCallaghan, D. M., FitzGerald, R. J., Donnelly, W. J., and Dalton, J. P. (1994). Proteolytic and peptidolytic activities in commercial pancreatic protease preparations and their relationship to some whey protein hydrolysate characteristics. **Journal of Agricultural and Food Chemistry.** 42: 2973-

2981.

Nagasawa, T., Yonekura, T., Nishizawa, N., and Kitts, D. D. (2001). *In vitro* and in *vivo* inhibition of muscle lipid and protein oxidation by carnosine. **Molecular and Cellular Biochemistry**. 225: 29-34.

Nawar, W. W. (1996). Lipids. In O. R. Fennema (Ed.). **Food chemistry** (pp. 225-319). New York: Marcel Dekker.

Ngo, D. H., Qian, Z. J., Ryu, B., Park, J. W., and Kim, S. K. (2010). *In vitro* antioxidant activity of a peptide isolated from Nile tilapia (*Oreochromis niloticus*) scale gelatin in free radical-mediated oxidative systems. **Journal of Functional Foods.** 2: 107-117.

Ou, B., Hampsch-Woodill, M., and Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. **Journal of Agricultural and Food Chemistry.** 49: 4619-4626.

Packer, J. E., Slater, T. F., and Wilson, R. L. (1979). Direct obser-vation of a free radical interaction between vitamin E and vitamin C. **Nature.** 278: 737-739.

Pangsorn, S., Laong-manee, P., and Siriraksophon, S. (2007). **Status of Surimi Industry in the Southeast Asia**. Southeast Asian Fisheries Development Center, Samutprakan, Thailand.

Pappenheimer, J. R. and Volpp, K. (1992). Transmucosal impedance of small intestine correlation with transport of sugars and amino acids. **American Journal of Physiology.** 263: C480-493.

Park, P. J., Jung, W. K., Nam, K. S., Shahidi, F., and Kim, S. K. (2001). Purification and characterization of antioxidative peptides from protein hydrolysate of

lecithin-free egg yolk. **Journal of the American Oil Chemists Society.** 78: 651-656.

Park, J. W. and Morrissey, M. T. 2000. Manufacturing of surimi from light muscle fish. In J. W. Park (Ed.). In **Surimi and surimi seafood** (pp. 23-58). New York: Marcel Dekker.

Peña-Ramos, E. A. and Xiong, Y. (2001). Antioxidative activity of whey protein hydrolysates in a liposomal system. **Journal of Dairy Science**. 84: 2577-2583.

Peng, X., Xiong, Y. L., and Kong, B. (2009). Antioxidant activity of peptide fractions from whey protein hydrolysates as measured by electron spin resonance. **Food Chemistry.** 113: 196-201.

Phanturat, P., Benjakul, S., Visessanguan, W., and 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.

Phelan, M., Aherne-Bruce, S. A., O'Sullivan, D., FitzGerald, R. J., and O'Brien, N. M. (2009). Potential bioactive effects of casein hydrolysates on human cultured cells. **International Dairy Journal**. 19: 279-285.

Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. **International Dairy Journal.** 16: 1306-1314.

Picardo, M. and Passi, S. (1997). Free radicals. In J. D. Bos (Ed.). **Skin immune system** (SIS) (pp. 207-226.). Boca Raton, New York: CRC Press.

Piyadhammaviboon, P. and Yongsawatdigul, J. (2009). Protein cross-linking ability of sarcoplasmic proteins extracted from threadfin bream. **LWT-Food Science and Technology.** 42: 37-43.

Piyadhammaviboon, P. and Yongsawatdigul, J. (2010). Proteinase inhibitory activity of sarcoplasmic proteins from threadfin bream (*Nemipterus* spp.). **Journal of Agricultural and Food Chemistry**. 90: 291-298.

Ponsoda, X., Jover, R., Castell, J., and Gomez-Lechon, M. (1991). Measurement of intracellular LDH activity in 96-well cultures: A rapid and automated assay for cytotoxicity studies. **Journal of Tissue Culture Methods.** 13: 21-24.

Prior, R. L., Wu, X., and Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements.(2005). **Journal of Agricultural and Food Chemistry**. 53: 4290-4302.

Qian, Z. J., Jung, W. K., Byun, H. G., and Kim, S. K. (2008a). Protective effect of an antioxidative peptide purified from gastrointestinal digests of oyster, *Crassostrea gigas* against free radical induced DNA damage. **Bioresource Technology.** 99: 3365-3371.

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

Raa, J. and Gildberg, A. (1982). Fish silage: a review. **Critical Reviews in Food Science and Nutrition.** 16: 383-419.

Raghavan, S., Kristinsson, H. G., and Leeuwenburgh, C. (2008). Radical scavenging and reducing ability of tilapia (*Oreochromis niloticus*) protein hydrolysates. **Journal of Agricultural and Food Chemistry**. 56: 10359-10367.

Rajapakse, N., Mendis, E., Byun, H. G., and Kim, S. K. (2005a). Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-

mediated oxidative systems. **The Journal of Nutritional Biochemistry.** 16: 562-569.

Rajapakse, N., Mendis, E., Jung, W. K., Je, J. Y., and Kim, S. K. (2005b). Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. **Food Research International.** 38: 175-182.

Ranathunga, S., Rajapakse, N., and Kim, S. K. (2006). Purification and characterization of antioxidative peptide derived from muscle of conger eel (*Conger myriaster*). **European Food Research and Technology.** 222: 310-315.

Rawlings, N. D. and Barrett, A. J. (1994). Families of serine peptidases. **Methods in Enzymology**. 244:19-61.

Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., and Cui, C. (2008). Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry. **Food Chemistry.** 108: 727-736.

Robyt, J. F. and White, B. J. (1987). **Biochemical Techniques Theory and Practics**. Brooks/Cole Publishing Co., CA., USA., pp: 120.

Rival, S. G., Boeriu, C. G., and Wichers, H. J. (2001). Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition. **Journal of Agricultural and Food Chemistry.** 49: 295-302.

Sadat, L., Cakir-Kiefer, C., N'Negue, M. A., Gaillard, J. L., Girardet, J. M., and Miclo, L. (2011). Isolation and identification of antioxidative peptides from bovine α-lactalbumin. **International Dairy Journal**. 21: 214-221.

Saiga, A., Tanabe, S., and Nishimura, T. (2003). Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. **Journal of**

Agricultural and Food Chemistry. 51: 3661-3667.

Sakanaka, S. and Tachibana, Y. (2006). Active oxygen scavenging activity of egg-yolk protein hydrolysates and their effects on lipid oxidation in beef and tuna homogenates. **Food Chemistry.** 95: 243-249.

Sakanaka, S., Tachibana, Y., Ishihara, N., and Juneja, L. R. (2004). Antioxidant activity of egg-yolk protein hydrolysates in a linoleic acid oxidation system. **Food Chemistry**. 86: 99-103.

Samaranayaka, A. G. P., Kitts, D. D., and Li-Chan, E. C. Y. (2010). Antioxidative and Angiotensin-I-Converting Enzyme Inhibitory Potential of a Pacific Hake (*Merluccius productus*) Fish Protein Hydrolysate Subjected to Simulated Gastrointestinal Digestion and Caco-2 Cell Permeation. **Journal of Agricultural and Food Chemistry.** 58: 1535-1542.

Samaranayaka, A. G. P. and Li-Chan, E. C. Y. (2011). Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. **Journal of Functional Foods.** 3: 229-254.

Sampath Kumar, N. S., Nazeer, R. A., and Jaiganesh, R. (2011). Purification and biochemical characterization of antioxidant peptide from horse mackerel (*Magalaspis cordyla*) viscera protein. **Peptides**. 32: 1496-1501.

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

Sarkar, A., Bishayee, A., and Chatterjee, M. (1995). Beta-carotene prevents lipid peroxidation and red blood cell membrane protein damage in experimental hepatocarcinogenesis. **Cancer Biochemistry Biophysics.** 15:111-25.

Savoie, L. Gauthier, S. F. Marin, J., and Pouliot, Y. (2005). *In vitro* determination of the release kinetics of peptides and free amino acids during the digestion of food proteins. **Journal of AOAC International**. 88: 935-948.

Schantz, M., Mohn, C., Baum, M., and Richling, E. (2010). Antioxidative efficiency of an anthocyanin rich bilberry extract in the human colon tumor cell lines Caco-2 and HT-29. **Journal of Berry Research.** 1: 25-33.

Sheih, I. C., Fang, T. J., and Wu, T. K. (2009a). Isolation and characterisation of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from the algae protein waste. **Food Chemistry.** 115: 279-284.

Sheih, I. C., Wu, T. K., and Fang, T. J. (2009b). Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems. **Bioresource Technology**. 100: 3419-3425.

Sies, H. (1993). Strategies of antioxidant defense. **European journal of biochemistry**. 215: 213-219.

Simmonds, N. J., and Rampton, D. S. (1993). Inflammatory bowel disease-a radical view. **Gut.** 34: 865-868.

Simpson, B. K. (2000). Digestive proteinases from marine animals. In N. M., Haard, and B. K. Simpson (Eds.). **Seafood enzymes: Utilization and influence on postharvest seafood quality** (pp. 531-540). New York: Marcel Dekker.

Sinsuwan, S., Rodtong, S., and Yongsawatdigul, J. (2008). Production and characterization of NaCl-activated proteinases from *Virgibacillus* sp. SK33 isolated from fish sauce fermentation. **Process Biochemistry.** 43:185-192.

Sinsuwan, S., Rodtong, S., and Yongsawatdigul, J. (2010). A NaCl-stable serine proteinase from V*irgibacillus* sp. SK33 isolated from Thai fish sauce. **Food**

Chemistry. 119: 573-579.

Suetsuna, K., Ukeda, H., and Ochi, H. (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. **Journal of Nutritional Biochemistry.** 11: 128-131.

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

Sun, Y., Pan, D., Guo, Y., and Li, J. (2012). Purification of chicken breast protein hydrolysate and analysis of its antioxidant activity. **Food and Chemical Toxicology**. 50: 3397-3404.

Tang, C. H., Peng, J., Zhen, D. W., and Chen, Z. (2009). Physicochemical and antioxidant properties of buckwheat (*Fagopyrum esculentum Moench*) protein hydrolysates. **Food Chemistry**. 115: 672-678.

Thiansilakul, Y., Benjakul, S., and Shahidi, F. (2007). Antioxidative activity of proteinhydrolysate from round scad muscle using Alcalase and Flavourzyme. **Journal of Food Biochemistry.** 31: 266-287.

Tironi, V. A. and Anón, M. C. Amaranth proteins as a source of antioxidant peptides: Effect of proteolysis. **Food Research International**. 43: 315-322.

Valko, M., Izakovic, M., Mazur, M., Rhodes, C. J., and Telser, J. (2004). Role of oxygen radicals in DNA damage and cancer incidence. **Molecular and Cellular Biochemistry**. 266: 37-56.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. **The International Journal of Biochemistry and Cell Biology.**

39: 44-84.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., and Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. **Chemico-Biological Interactions.** 160: 1-40.

Valko, M., Morris, H., and Cronin, M. T. D. (2005). Metals, toxicity and oxidative stress. **Current Medicinal Chemistry.** 12: 1161-1208.

Van der Ven, C., Gruppen, H., de Bont, D. B. A., and Voragen, A. G. J. (2002). Correlations between biochemical characteristics and foam-forming and-stabilizing ability of whey and casein hydrolysates. **Journal of Agricultural and Food Chemistry.** 50: 2938-2946.

Vercruysse, L., Smagghe, G., Beckers, T., and Van Camp, J. (2009). Antioxidative and ACE inhibitory activities in enzymatic hydrolysates of the cotton leafworm, *Spodoptera littoralis*. **Food Chemistry.** 114: 38-43.

Vercruysse, L., Van Camp, J., and Smagghe, G. (2005). ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: A Review. **Journal of Agricultural and Food Chemistry.** 53: 8106-8115.

Vitaglione, P., Morisco, F., Caporaso, N., and Fogliano, V. (2004). Dietary antioxidant compounds and liver health. **Critical Reviews in Food Science and Nutrition.** 44: 575-586.

Wang, H. and Joseph, J. A. (1999). Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. **Free Radical Biology and Medicine**. 27: 612-616.

Wang, B., Li, L., Chi, C. F., Ma, J. H., Luo, H. Y., and Xu, Y. f. (2013). Purification and characterisation of a novel antioxidant peptide derived from blue mussel

(*Mytilus edulis*) protein hydrolysate. **Food Chemistry.** 138: 1713-1719.

Wang, D., Wang, L. J., Zhu, F. X., Zhu, J. Y., Chen, X. D., and Zou, L. (2008). *In vitro* and *in vivo* studies on the antioxidant activities of the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food). **Food Chemistry**. 107: 1421-1428.

Wibowo, S., Savant, V., Cherian, G., Savage, T. F., and Torres, J. A. (2005). Evaluation as a feed ingredient of surimi wash water protein recovered using a chitosan–alginate complex. **Journal of Aquatic Food Product Technology.** 14: 55-72.

Wichi, H. P. (1988). Enhanced tumor development by butylated hydroxyanisole (BHA) from the oseophageal squamous epithelium. **Food and Chemical Toxicology.** 26: 717-723.

Wu, H. C., Chen, H. M., and Shiau, C. Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). **Food Research International.** 36: 949-957.

Xia, Y. C., Bamdad, F., Ganzle, M., and Chen, L. Y. (2012). Fractionation and characterization of antioxidant peptides derived from barley glutelin by enzymatic hydrolysis. **Food Chemistry.** 134: 1509-1518.

Xie, Z., Huang, J., Xu, X., and Jin, Z. (2008). Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate. **Food Chemistry.** 111: 370-376.

Yang, J. L., Ho, H. Y., Chu, Y. J., and Chow, C. J. (2008). Characteristic and antioxidant activity of retorted gelatin hydrolysates from cobia (*Rachycentron canadum*) skin. **Food Chemistry.** 110: 128-136.

Yongsawatdigul, J., Worratao, A., and Park, J. W. (2002). Effect of endogenous

transglutaminase on threadfin bream surimi gelation. **Journal of Food Science**. 67: 3258-3263.

You, L., Zhao, M., Cui, C., Zhao, H., and Yang, B. (2009). Effect of degree of hydrolysis on the antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysates. **Innovative Food Science and Emerging Technologies.** 10: 235-240.

You, L., Zhao, M., Regenstein, J. M., and Ren, J. (2010). Changes in the antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysates during a simulated gastrointestinal digestion. **Food Chemistry.** 120: 810-816.

You, L., Zhao, M., Regenstein, J. M., and Ren, J. (2011). *In vitro* antioxidant activity and *in vivo* anti-fatigue effect of loach (*Misgurnus anguillicaudatus*) peptides prepared by papain digestion. **Food Chemistry.** 124: 188-194.

Young, D., Nau, F., Pasco, M., and Mine, Y. (2011). Identification of hen egg yolk-derived phosvitin phosphopeptides and their effects on gene expression profiling against oxidative stress-induced Caco-2 Cells. **Journal of Agricultural and Food Chemistry.** 59: 9207-9218.

Yu, B. P. (1994): Cellular defenses against damage from reactive oxygen species. **Physiological Reviews.** 74: 139-162.

Zhang, Y., Duan, X., and Zhuang, Y. (2012). Purification and characterization of novel antioxidant peptides from enzymatic hydrolysates of tilapia (*Oreochromis niloticus*) skin gelatin. **Peptides.** 38: 13-21.

Zhang, T., Li, Y., Miao, M., and Jiang, B. (2011). Purification and characterisation of a new antioxidant peptide from chickpea (*Cicer arietium* L.) protein hydrolysates. **Food Chemistry.** 128: 28-33.

Zhang, S., Wang, Z., Xu, S., and Gao, X. (2009). Purification and characterization of a radical scavenging peptide from rapeseed protein hydrolysates. **Journal of the American Oil Chemists' Society.** 86: 959-966.

Zhang, J. H., Zhang, H., Wang, L., Guo, X. N., Wang, X. G., and Yao, H. Y. (2010). Isolation and identification of antioxidative peptides from rice endosperm protein enzymatic hydrolysate by consecutive chromatography and MALDI-TOF/TOF MS/MS. **Food Chemistry.** 119: 226-234.

Zhao, X. and Hou, Y. (2009). Limited hydrolysis of soybean protein concentrate and isolate with two proteases and the impact on emulsifying activity index of hydrolysates. **African Journal of Biotechnology**. 8: 3314-3319.

Zheng, X. Q., Li, L. T., Liu, X. L., Wang, X. J., Lin, J., and Li, D. (2006). Production of hydrolysate with antioxidative activity by enzymatic hydrolysis of extruded corn gluten. **Applied Microbiology and Biotechnology.** 73: 763-770.

Zhou, D. Y., Zhu, B. W., Qiao, L., Wu, H. T., Li, D. M., Yang, J. F., and Murata, Y. (2012). *In vitro* antioxidant activity of enzymatic hydrolysates prepared from abalone (*Haliotis discushannai* Ino) viscera. **Food and Bioproducts Processing.** 90:148-154.

Zhu, K., Zhou, H., and Qian, H. (2006). Antioxidant and free radical-scavenging activities of wheat germ protein hydrolysates (WGPH) prepared with alcalase. **Process Biochemistry**. 41: 1296-1302.

CHAPTER III

ANTIOXIDANT ACTIVITY OF PROTEIN HYDROLY-SATES DERIVED FROM THREADFIN BREAM SURIMI BYPRODUCTS

3.1 Abstract

Antioxidant activities of protein hydrolysates from threadfin bream surimi wastes, including frame, bone and skin (FBS) and refiner discharge (RD), were investigated. FBS and RD were rich in Lys, Glu, Gly, Pro, and Phe. FBS was hydrolyzed to a greater extent than RD regardless of proteinases tested (*Virgibacillus*sp. SK33 proteinase, Alcalase, pepsin and trypsin). Pepsin-hydrolyzed FBS at 5% degree of hydrolysis (DH) showed the highest antioxidant activity based on 2, 2'-azinobis (3-ethyl-benzothiazoline-6-sulfonate) (ABTS) radical (0.455±0.054 mg Trolox equivalents/mg leucine equivalents), ferric reducing antioxidant power (FRAP) (0.221±0.005 mM Trolox equivalents) and inhibition of β-carotene bleaching assays. FBS hydrolysates showed higher antioxidant activity based on chemical assays than their RD counterparts. However, FBS and RD hydrolysates protected HepG2 cells against *tert*-butyl hydroperoxide-induced oxidative damage to a similar extent. Therefore, FBS and RD hydrolysates have a potential as antioxidative neutraceutical ingredients.

Keywords: Surimi wastes, protein hydrolysates, antioxidant activity, HepG2 cell line

3.2 Introduction

Thailand is one of the largest surimi producers in Southeast Asia. Surimi production increased from 65,000 MT in 1978 to about 150,000 MT in 2005 (Pangsorn, Laong-manee, and Siriraksophon, 2007). Threadfin bream (*Nemipterus* spp.) is a major raw material for surimi production in Thailand. The quantity of threadfin bream raw materials supplied to surimi production is estimated to be 200,000 MT/year (Pangsorn et al., 2007). Normally, the solid wastes from deboning machines and a refining process are about 22-27 and 5%, respectively (Morrisey, Lin, andIsmond, 2005). It is, therefore, estimated that frame and skin/bone as well as refiner discharges from threadfin bream surimi production are about 50,000-60,000 MT annually. These wastes are usually converted to low value fish meal. A means to fully utilize and increase marketability of these wastes should be sought.

Protein hydrolysates have recently been reported to inhibit lipid oxidation in food and cellular systems (Park, Jung, Nam, Shahidi, and Kim, 2001). Antioxidant activity of protein hydrolysates mainly relies on peptides present in the hydrolysate. Hydrolysates rich in peptides containing hydrophobic amino acids, such as Pro, Leu, Ala, Trp and Phe, are believed to possess high antioxidant activity (Mendis, Rajapakse, and Kim, 2005). Peptides containing Trp, Tyr and Met also show the highest antioxidant activity, followed by Cys, His and Phe (Dávalos, Miguel, Bartolomé, and López-Fandiño, 2004). In addition, acidic (Asp and Glu) and/or basic (Lys) amino acids play an important role in the chelation of metal ions by carboxyl and amino groups in their side chains (Rajapakse, Mendis, Byun, and Kim, 2005). Therefore, amino acid composition of peptides in protein hydrolysates is a critical factor in controlling the antioxidant activity of protein hydrolysates.

Amino acid composition of peptides in protein hydrolysate is entirely dependent on the protein substrates and proteinases used. Thus far, commercial proteinases including pepsin, trypsin, and Alcalase have been reported to produce protein hydrolysates with antioxidant activity (Qian, Jung, and Kim, 2008). The use of proteinases from novel sources in protein hydrolysate production has not been widely studied. *Virgibacillus* sp. SK33 isolated from one-month-old Thai fish sauce mashes is a potential source of extracellular proteinases with broad specificity toward oxidized insulin B with the dominant cleavage sites at Tyr^{16}-Leu^{17} and Phe^{25}-Tyr^{26} (Sinsuwan, Rodtong, and Yongsawatdigul, 2010). Peptides containing amino acid residues exhibiting antioxidant activity would be obtained through hydrolytic reaction of *Virgibacillus* sp. SK33 proteinases. This could be a potential processing-aid for production of bioactive protein hydrolysate with antioxidant activity.

Antioxidant activity based on radical scavenging assays has some limitations (Alamed, Chaiyasit, McClements, and Decker, 2009). Most of these assays including ABTS and FRAP assays are conducted under non-physiological conditions. For this reason, the results might not necessarily correlate with biological responses. However, radical scavenging assays are simple, inexpensive, rapid and helpful in evaluating the antioxidant mechanisms of a compound. A step closer to *in vivo* is the use of the cellular model system. Therefore, antioxidant activity of a compound of interest should be tested in both radical scavenging assays and in cellular system.

The use of normal human hepatocytes, which closely represent the parent cell types, is restricted due to limited growth potential, expensive, legal and ethical considerations. Under proper culture, the human hepatocarcinoma HepG2 cell line can display highly morphological and functional differentiation resembling normal human

hepatocytes (Narayanan, Fitch, and Levenson, 2001). Therefore, the cell line has been widely used in various in *vitro* model systems including the antioxidant activity of compounds toward liver.

The objectives of this study were to produce protein hydrolysates from threadfin bream surimi byproducts, FBS and RD, using *Virgibacillus* sp. SK33 and commercial proteinases including Alcalase, pepsin and trypsin. In addition, antioxidant activities of protein hydrolysates were also determined based on proton/electron donating ability in aqueous solution (ABTS radical scavenging and FRAP assays) and proton donating in emulsion systems (inhibition of β-carotene bleaching) in comparison with the cytoprotective effect on *tert*-butyl hydroperoxide (TBHP) in HepG2 cells.

3.3 Materials and methods

3.3.1 Materials

Surimi byproducts including FBS and RD were obtained from Andaman Surimi Industry (Samutsakorn, Thailand). Samples were packed in a polystyrene foam box filled with ice and transported to a laboratory at Suranaree University of Technology. Upon arrival, FBS and RD were dried at 75°C for 5 h, and ground using an IKA milling machine (IKA M20, IKA-Werke GmbH & Co, Staufen, Germany). Ground samples were passed through a 2-mm sieve. Dried powders were defatted using isopropanol with a ratio of powder to solvent of 1: 3 at 40 °C for 20 min, which was repeated a total of three times. Defatted samples were left at room temperature for 2 h, vacuum-packed and kept at -20°C until used. Alcalase 2.4 L was gifted from Novozymes (Bagsvaerd, Denmark). Trypsin from hog pancreas, 2, 2’-azino-bis (3-

ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were purchased from Biochemika (Buchs, Switzerland). Pepsin from porcine stomach mucosa, trinitrobenzenesulfonic acid (TNBS) and 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HepG2 human hepatoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle Media (DMEM) was purchased from Gibco BRL, Life Technologies (Gaithersburg, MD, USA). Other chemicals and reagents used were of analytical grade.

3.3.2 *Vigibacillus* sp. SK 33 proteinases production

Inoculum was prepared by transferring a loopful of pure culture of *Virgibacillus* sp. SK33 into Y-broth (5% NaCl, 1% yeast extract, 0.3% trisodium citrate, 0.2% KCl and 2.5% $MgSO_4 \cdot 7H_2O$, pH 7.0) and incubated at 35°C for 1 day (Sinsuwan, Rodtong, and Yongsawatdigul, 2008). The inoculum optical density (OD) measured at 600 nm was about 0.3 which was equivalent to cell counts of 10^7 CFU/ml. Fifty ml of inoculum were transferred to 450 ml of the culture medium containing 2.5% NaCl, 1% brewer yeast sludge, 0.3% trisodium citrate, 0.2% KCl and 2.5% $MgSO_4 \cdot 7H_2O$, pH 8.0 and incubated at 40°C with a shaking speed of 100 rpm for 3 day. Crude extracellular proteinase was collected by centrifugation at 10,000 g, 4°C for 10 min. Supernatants were collected and filtered through a 0.45-µm membrane. Proteinase activity was assayed following the method of Sinsuwan et al. (2008) using Suc-Ala-Ala-Pro-Phe-aminomethylcoumarin (AMC) as substrate. Unit activity was defined as the amount of released AMC in nanomole per min. Then, crude enzyme was lyophilized and kept at 4°C throughout the study.

3.3.3 Chemical properties of defatted FBS and RD powder

3.3.3.1 Proximate composition and mineral contents

Moisture, fat, protein, and ash of defatted FBS and RD powder were determined according to AOAC (2000). Mineral contents, namely calcium (Ca), phosphorus (P), iron (Fe), sodium (Na), magnesium (Mg), and potassium (K), of defatted FBS and RD powder were determined using inductively coupled plasma emission spectrometry (ICP-OES, Model Optima 4300 DV, Perkin-Elmer, USA) according to the method of AOAC (2000). Dried samples (0.5 g) were digested in 9 ml of concentrated nitric acid and 3 ml of hydrofluoric acid using microwave heating at 180±5°C for 15 min. Wavelengths for the determination of Ca, Mg, Na, P, K and Fe were 317.9, 285.2, 588.9, 213.6, 766.4 and 248.3 nm, respectively.

3.3.3.2 Amino acid analysis by GC-MS

Defatted FBS and RD powder were hydrolyzed following AOAC (2000). In brief, 0.05-0.10 g of samples was mixed with performic acid for 16 h in an ice bath in order to oxidize cystine and methionine to cysteic acid and methionine sulfone, respectively. Consequently, sodium metabisulfite was added to decompose the performic acid. Subsequently, samples were digested with 6 N HCl at 110 °C for 24 h under nitrogen. The digested samples were derivertized with propyl chloroformate. Quantitative analysis of total amino acids was achieved by gas chromatography–mass spectrometry (GC–MS, Agilent 6890N GC/5973 Inert MSD, Agilent Technologies, Darmstadt, Germany) equipped with a ZB-AAA column (10 m x 0.25 mm I.D., 0.25 µm film thicknesses, Phenomenex, Torrance, USA). The amino acid was expressed as g/100 g protein.

3.3.3.3 Estimation of quality of the amino acids

The amino acid score for the essential amino acids was calculated following the equation (FAO/WHO (1973)):

$$\text{Amino acid score} = \frac{\text{Amount of amino acid per sample protein [mg/g]} \times 100}{\text{Amount of amino acid per protein in reference protein [mg/g]}}$$

3.3.3.4 SDS-PAGE pattern

SDS-PAGE was performed according to Laemmli (1970). One gram of defatted dry powder of FBS and RD were solubilized in 19 ml of 8 M urea and homogenized using an IKA homogenizer (IKA Works Asia, Bhd, Malaysia) for 5 min, and centrifuged at 4 ˚C, 10,000 g for 20 min. Supernatants were dialyzed using a dialysis membrane with molecular weight cut-off 100 Da against distilled water in order to remove urea. Protein (30 µg) was loaded onto 15% acrylamide gel. Gels were run at a constant voltage at 120 V, stained with 0.125% Coomassie brilliant blue R-250 and destained in a solution containing 25% ethanol and 10% acetic acid.

3.3.4 Production of FBS and RD hydrolysates

Ten grams of defatted FBS and RD powder in 90 ml of 0.1 M sodium phosphate buffer were hydrolyzed using various enzymes under their optimal condition: pH 8, 65 °C for *Virgibacillus* sp. SK33 proteinase; pH 8, 60 °C for Alcalase; pH 2, 37 °C for pepsin and pH 8, 37 °C for trypsin. The ratio of commercial proteinases to substrate was 1: 100 (w/w). *Virgibacillus* sp. SK33 proteinase was added to hydrolysate mixture at final unit activity of 13.5 U per gram samples.

Samples were hydrolyzed for 0, 2, 4, 8, 12 and 24 h. Subsequently, they were heated at 90 °C for 10 min to terminate the reaction and centrifuged at 10,000 g for 20 min at 4 °C. The supernatants were referred to as FBS and RD hydrolysates. The peptide content was determined using the TNBS method (Adler-Nissen, 1979).

3.3.5 Properties of FBS and RD hydrolysates

3.3.5.1 Degree of hydrolysis (DH)

DH was performed according to Adler-Nissen (1979). Fifty µl of each hydrolysate were mixed with 0.5 ml of 0.2125 M phosphate buffer, pH 8.2 and 0.5 ml of 0.05% TNBS reagent. The mixture was incubated at 50 °C for 1 h in a water bath. One ml of 0.1 N HCl was added to stop reaction and left at room temperature for 30 min. Absorbance was monitored at 420 nm. Leucine was used as a standard. To determine total amino acid content, defatted powder sample was hydrolyzed with 6 N HCl (samples to acid ratio of 1: 100) at 120 °C for 24 h using an autoclave. The degree of hydrolysis was calculated following the equation:

$$\%DH = ([(L_t - L_0)/ (L_{max} - L_0)] \times 100$$

Where L_t was the amount of α-amino acid released at time *t*. L_0 was the amount of α-amino acid in the supernatant at 0 h. L_{max} was the total amount of α-amino acid obtained after acid hydrolysis.

Amino acid composition of FBS and RD hydrolysates was also determined as described in 3.3.3.2.

3.3.5.2 Antioxidant activity of FBS and RD hydrolysates

3.3.5.2.1 ABTS radical scavenging activity assay

ABTS radical scavenging activity assay was performed according to Arnao, Cano, and Acosta (2001) with some modifications. $ABTS^{\bullet+}$ stock solution

was prepared by mixing 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution in 10 mM phosphate buffer pH 7.4, and kept in the dark for 16 h. Fresh $ABTS^{\bullet+}$ working solution was prepared by mixing $ABTS^{\bullet+}$ stock solution in 10 mM phosphate buffer pH 7.4 to attain absorbance of 0.7 ± 0.02 at 734 nm. Twenty µl of hydrolysates obtained from 8-h hydrolysis were mixed with 1980 µl of fresh $ABTS^{\bullet+}$ working solutions. Then, the reaction mixture was kept in the dark for 5 min and absorbance was monitored at 734 nm. Trolox (0.5-2.5 mM) was used as standard. Results were expressed as mg Trolox equivalents/mg leucine equivalents.

3.3.5.2.2 Ferric reducing antioxidant power assay (FRAP assay)

FRAP assay was performed according to Benzie and Strain (1996) with some modifications. FRAP reagent was prepared by mixing 25 ml of 300 mM acetate buffer, pH 3.6, 2.5 ml of 10 mM TPTZ solution in 40 mM HCl, and 2.5 ml of 20 mM $FeCl_3 6\ H_2O$ solution. One hundred µl of 1.43 mg leucine equivalent/ml of hydrolysates obtained from 8-h hydrolysis were mixed with 1 ml of fresh FRAP reagent. Then, the solution was incubated at 37 °C for 15 min in a water bath. Absorbance of each sample was monitored at 593 nm. Trolox (0.05-0.4 mM) was used as standard. Results were expressed as mM Trolox equivalents.

3.3.5.2.3 Inhibition of ß-carotene bleaching assay

β-carotene bleaching assay was performed according to Koleva, van Beek, Linssen, de Groot, and Evstatieva (2002) with some modifications. β-Carotene-linoleic acid emulsion was prepared by mixing 0.5 mg β-carotene in 1 ml chloroform, 25 µl of linoleic acid and 200 µl of Tween-40. Chloroform was evaporated under vacuum at 45 °C and 100 ml of distilled water were added. One

hundred µl of 1.26 mg leucine equivalent/ml of hydrolysates obtained from 8-h hydrolysis were mixed with 2 ml of flesh β-carotene-linoleic acid emulsion. Then, the mixture was incubated at 50 °C for 2 h in a water bath. Absorbance was monitored at 470 nm. BHA (0.01%) was used as a positive control.

3.3.5.3 Determination of cytotoxicity/cytoprotective effect

3.3.5.3.1 Cell culture

HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), HEPES buffer pH 7.4 (0.015 M), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were incubated in a humidified incubator containing 5% CO_2 and 95% air at 37 °C and subcultured by trypsinisation when they reached confluence, and treated as described below.

3.3.5.3.2 Determination of TBHP-induced cytotoxicity on HepG2 cells

Cells were plated in a 96-well plate (3.0×10^4 cells per well) and incubated in a humidified incubator containing 5% CO_2 and 95% air at 37 °C for 24 h. Then, the cells were treated with various concentrations of TBHP (0-1200 µM) and incubated for 2 h. The culture medium was removed and replaced with a fresh medium containing 100 µl of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and further incubation for 4 h. Subsequently, the cells were centrifuged at 2,000 rpm at 4 °C for 5 min. The culture medium was discarded and the purple formazan crystals were dissolved with DMSO (100 µl/well). Absorbance was monitored at 590 nm using a microplate reader (Benchmark plus, Bio-Rad, Tokyo, Japan). The result was expressed as percentage of viable cells compared to the control culture (without TBHP).

3.3.5.3.3 Protective effect of hydrolysates on TBHP-induced cytotoxicity

Cells were plated in a 96-well plate (3.0×10^4 cells per well) and incubated in a humidified incubator containing 5% CO_2 and 95% air at 37 °C for 24 h. Then, cells were pretreated with 12.5 µg leucine equivalent/ml of each hydrolysate and incubated for 24 h. The culture medium was removed and replaced with a fresh medium containing 500 µM TBHP and further incubated for 2 h. Subsequently, cell viability was determined using MTT dye solution as described in section 3.3.5.3.2. The result was expressed as a percentage of viable cells compared to the control culture (without TBHP).

3.3.6 Statistical analyses

Hydrolysis experiments were repeated twice using 2 different lots of FBS and RD (*n*=2). All the tests were done in duplicate. Data were expressed as means with standard deviations. Data were analyzed using analysis of variance (ANOVA) and means comparison was performed using Duncan's multiple range test (DMRT) at 95% confidence level using a SPSS package (SPSS 17.0 for Windows, SPSS Inc, Chicago, IL, USA).

3.4 Results and discussion

3.4.1 Chemical characteristics of defatted FBS and RD powder

Crude protein of FBS and RD was 41.70±2.68 and 66.30±2.63% (d. b.), respectively (Table 3.1). FBS contained a high amount of inorganic matter as it contained bones. Relatively high content of ash was also found in RD. High content of Ca and P in RD suggested that solid discharges from the refining process also

contained bones. Kim and Park (2005) reported that refiner discharge of Pacific Whiting from surimi processing consisted of muscle at 95%, skin at 2.1%, bone at 2.9%, and scale fragment at a trace amount. Proximate composition indicated that these byproducts are a rich source of protein that can be utilized by a more efficient means to produce more value added products. The results of mineral content revealed that Ca and P were major inorganic matters in FBS and RD powder (Table 3.1).

FBS and RD were rich in Lys, Glu, Gly, Pro, Phe, Leu, His, Tyr and Asp (Table 3.2). Amino acid profiles of FBS were slightly different from those of RD. Compared to RD, FBS contained higher amounts of Glu, Lys, Phe and Tyr but lower amounts of Gly, Pro, His and Asp. The total essential amino acid contents in FBS and RD were 69.2 and 66.2 g/100 g protein, respectively. These values are higher than the recommended reference (33.9 g/100 g protein) (Table 3.3). Based on the result of the amino acid profiles, FBS and RD are good sources of essential amino acids, especially Lys, Leu, His, Phe and Tyr. Moreover, the amino acid scores of FBS and RD were compared with FAO/WHO (1991) standard (Table 3.3). Results show that Ile, Leu, Met+Cys, Phe+Tyr, Trp and His were present in higher scores, whereas Thr and Val were less than the reference in both of FBS and RD. Thr was the limiting amino acid in FBS and RD samples. These results suggested that in order to fulfill the daily need for the essential amino acid in both of FBS and RD samples, it would require 100/46 or 2.2 times as much FBS protein and 100/41 or 2.4 times as much RD protein to be consumed when they are the sole protein in the diet. Fish flesh and skin of herring (*Clupea harengus*,), Atlantic mackerel (*Scomber scombrus*), horse mackerel (*Trachurus trachurus*) and white hake (*Urophycis tenuis*) were rich in Lys, Glu, Asp, Gly, Ala, Pro, Met and Leu (Oluwaniyi, Dosumu, and Awolola, 2010). Collagens

from solid byproducts, frame, skin and refiner discharge from surimi production were also rich in Gly, Pro, Hyp-OH and Glu, but poor in Tyr, His, Met and Cys (Kim, and Park, 2004).

Table 3.1 Proximate compositions (% dry basis) and mineral contents (g/kg) of defatted FBS and RD powder

Compositions	**Content**	
	FBS	**RD**
Crude protein	41.7±2.68	66.3±2.63
Crude lipid	0.30±0.03	0.60±0.27
Moisture	2.80±1.00	5.10±0.06
Carbohydrate	7.30±0.74	5.30±1.98
Ash	47.90±1.86	22.70±0.98
Ca	146.95	50.39
P	72.65	23.69
Fe	0.02	0.01
Na	3.41	2.16
K	3.95	0.41
Mg	3.26	1.26

Note: FBS is the mixture of frame, bone and skin; RD is refiner discharges.

Table 3.2 Amino acid composition of defatted FBS and RD powder (g/ 100 g protein)

Amino acid	FBS	RD
Ala	1.65	1.49
Arg	0.11	0.09
Asp	4.91	6.49
Cys[a]	0.01	1.07
Glu	12.02	8.95
Gly	4.26	5.73
His[b]	7.36	8.58
Hyl	0.01	0.09
Hyp	1.76	3.18
Ile[a]	3.43	3.43
Leu[a]	7.05	7.18
Lys[a]	26.06	22.31
Met[a]	2.82	3.13
Phe[a]	8.17	7.35
Pro	4.94	6.28
Ser	1.19	1.49
Thr[a]	1.57	1.39
Typ[a]	2.08	1.91
Tyr[a]	7.79	6.79
Val[a]	2.81	3.06
TTHP	35.12	37.65
Total	100	100

Note: FBS is the mixture of frame, bone and skin; RD is refiner discharge; TTHP is total hydrophobic amino acid; [a]Essential amino acid according to FAO/WHO (1975); [b]Indispensable amino acid in human adult according to AO/WHO/ UNU (1985).

Table 3.3 Amino acids score (%) of defatted FBS and RD powder

Amino acid	FBS (g/100 g protein)	RD (g/100 g protein)	Standard FAO/WHO (1991) (g/100 g protein)	Score (%)	
				FBS	RD
Ile	3.43	3.43	2.8	123	123
Leu	7.05	7.18	6.6	107	109
Lys	26.06	22.31	5.8	449	385
Met+Cys	2.83	4.2	2.5	113	168
Phe+Tyr	15.96	14.14	6.3	253	224
Thr	1.57	1.39	3.4	**46**	**41**
Typ	2.08	1.91	1.1	189	174
Val	2.81	3.06	3.5	80	87
His	7.36	8.58	1.9	387	452
Total	69.15	66.2	33.9		

Note: FBS, and RD are the same as described in Table 3.2

3.4.2 SDS-PAGE pattern

FBS composed of major protein bands with molecular weight of 57, 44, 42, 38, 32, 27, 25, 23, 17 and 16 kDa while RD showed intense protein bands at about 38, 17 and 16 kDa. In addition, substantial amounts of low molecular weight proteins (<14 kDa) are observed in FBS and RD (Figure 3.1). Collagen extracted from frame, skin and the refiner discharge showed molecular weight at about 100 kDa (α1 and α2 chains) and 200 kDa (β-component) (Kim and Park, 2004). Suárez, Martínez, Sáez, Morales, and García-Gallego (2010) reported that sarcoplasmic proteins showed molecular weight of 97, 60, 51, 41, 36, 34, 27, and 25 kDa. Based on SDS-PAGE patterns, FBS and RD powders were likely derived from noncollagen muscle proteins,

namely myofibrillar and sarcoplasmic proteins, and degraded collagens.

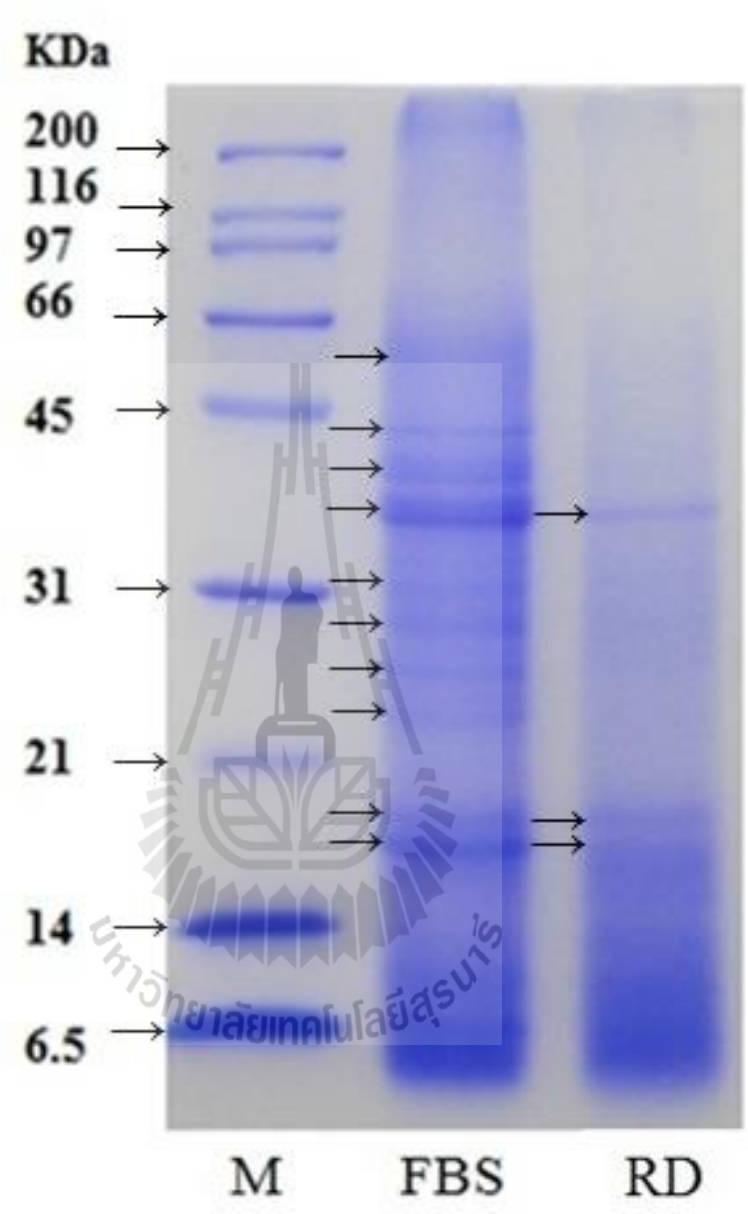

Figure 3.1 SDS-PAGE pattern of defatted FBS and RD powder. (M) Marker; (FBS) the mixture of frame, bone and skin; (RD) refiner discharges.

3.4.3 Degree of hydrolysis (DH)

DH of FBS and RD hydrolysates rapidly increased in the first 2 h, and reached plateau after 8 h of hydrolysis (Figures 3.2a and b). Trypsin-catalyzed reaction underwent spoilage after 8 h hydrolysis at 37 °C, pH 8.0.Therefore, it was terminated at 8 h. FBS was hydrolyzed by all studied proteinases to a greater extent than RD ($p<0.05$). This could be attributed to different availability of susceptible peptide bonds of these 2 substrates (Figure 3.1). In addition, Ca^{2+} has been reported to increase activity and stability of subtilisin superfamily, pepsins A and B, trypsin and *Virgibacillus* sp. SK33 proteinases against autolysis (Klomklao, Kishimura, Yabe, and Benjakul, 2007; Lu, Zhou, and Cai, 2008; Sinsuwan et al., 2010; Smith, Toogood, Baker, Daniel, and Baker, 1999). The higher Ca^{2+} content in FBS could probably increase activity and stability of proteinases, resulting in higher DH in FBS than RD.

The highest DH was found in hydrolysates prepared from Alcalase, followed by *Virgibacillus* sp. SK33 proteinase, trypsin and pepsin, respectively ($p<0.05$) at any hydrolysis time regardless of substrates (Figures 3.2a and b). DH of all hydrolysates reached plateau after 8 h of hydrolysis. Therefore, hydrolysates prepared under 8 h were selected for determination of antioxidant activity based on ABTS, FRAP, inhibition of β-carotene bleaching and its cytroprotective effect assays, and amino acid composition.

Amino acid composition revealed that FBS hydrolysates prepared from all proteinases at 8 h hydrolysis were richer in Lys, Tyr, His and hydrophobic amino acids including, Val, Ile, Leu and Phe, but poorer in Gly and Pro as compared to RD hydrolysates (Table 3.4). This was as expected because major amino acids of collagen, a main protein component of RD, are Gly and Pro.

FBS and RD hydrolysates prepared from Alcalase contained higher amounts of hydrophobic amino acids of Tyr, Phe, Leu, Ile, and Val than those prepared from other proteinases. In addition, they were rich in Asp, Glu, Gly and Lys. Alcalase is an endoproteinase having broad specificity, and high specificity toward aromatic (Phe, Trp, and Tyr), acidic (Glu), sulfur-containing (Met), aliphatic (Leu and Ala), hydroxyl (Ser), and basic (Lys) residues (Doucet, Otter, Gauthier, and Foegeding, 2003).

Proteinases from *Virgibacillus* sp. SK33 resulted in hydrolysates rich in Pro, Gly, Ala, Leu, Phe, Glu, and Lys. This result correlated with Sinsuwan et al. (2010) who reported that the purified *Virgibacillus* sp. SK33 proteinase showed broad specificity toward oxidized insulin B including Gln^{4}, Cys^{7}, Glu^{13}, Ala^{14}, $Leu^{15,17}$, $Tyr^{16,26}$, Arg^{22}, $Phe^{24,25}$, and Lys^{29}. As trypsin is specific for Lys at the carboxyl side of a peptide bond, trypsin-hydrolyzed samples contained high amount of Lys. FBS and RD prepared from trypsin were also rich in Asp, Glu, Gly, Leu, Phe and Pro. FBS and RD prepared from pepsin showed high content of Gly, Pro, Phe, Leu, Lys, Asp and Glu. Pepsin shows specificity toward aromatic amino acids: Phe; Tyr; and Trp (Simpson, 2000) and hydrophobic amino acids, such as Leu. These results indicated that amino acid composition of protein hydrolysate greatly varied with proteinase applied in the process.

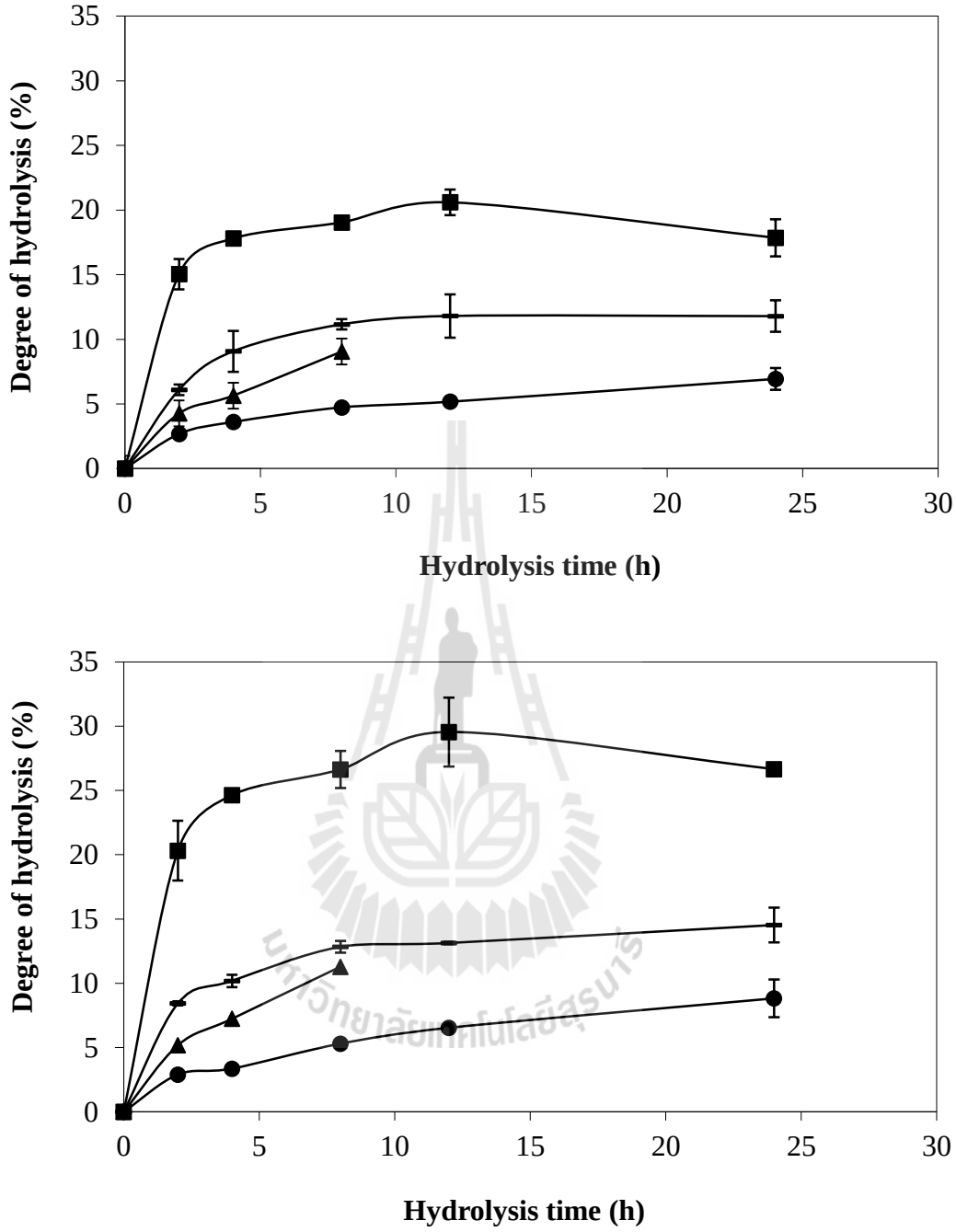

Figure 3.2 Degree of hydrolysis of defatted FBS (a) and RD hydrolysates (b) treated with various enzymes at different hydrolysis time. ( ) *Virgibacillus* sp. SK33; ( ) Alcalase; ( ) Pepsin; ( ) Trypsin.Values are expressed as means ± S.D. (n=2).

Table 3.4 Amino acid composition of defatted FBS and RD at 8 h. hydrolysis (g/100 g protein).

Amino	VH		AH		PH		TH	
acid	**FBS**	**RD**	**FBS**	**RD**	**FBS**	**RD**	**FBS**	**RD**
Ala	6.75	4.81	3.28	3.22	3.50	2.83	3.64	4.05
Arg	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Asp	5.01	5.11	5.64	6.13	5.53	5.45	5.79	5.71
Cys	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Glu	9.62	10.72	10.02	10.01	10.41	11.11	10.74	10.58
Gly	10.71	13.28	5.56	9.40	9.19	11.21	6.91	11.07
His	3.14	2.90	4.70	4.26	4.83	4.21	4.71	3.99
Hyl	0.02	1.92	1.40	1.99	2.24	2.93	1.52	2.69
Hyp	2.96	4.21	1.68	3.41	3.42	4.48	2.35	4.29
Ile	3.92	2.93	4.85	3.83	3.29	2.58	3.70	2.82
Leu	9.94	7.97	10.49	8.98	8.52	7.65	9.76	8.03
Lys	18.58	16.07	20.67	17.34	20.26	18.63	23.97	18.45
Met	3.06	2.62	3.21	2.95	2.59	3.06	2.51	2.60
Phe	7.70	7.26	8.28	7.47	7.17	6.96	6.93	6.63
Pro	9.10	11.69	5.69	8.25	8.17	9.49	6.13	9.53
Ser	1.56	1.25	1.31	1.42	1.40	1.41	1.23	1.44
Thr	1.33	1.12	1.51	1.67	1.38	1.37	1.41	1.46
Typ	0.02	0.15	1.25	0.72	0.40	0.27	0.59	0.33
Tyr	3.21	3.06	6.72	5.54	4.60	3.61	4.93	3.44
Val	3.30	2.90	3.71	3.41	3.06	2.75	3.16	2.86
TTHB	54.48	53.46	45.07	47.51	45.50	46.53	47.60	42.75
Total	100	100	100	100	100	100	100	100

Note: FBS, RD, TTHP are the same as described in Table 3.2. VH, AH, PH and TH is hydrolysate prepared from *Virgibacillus* sp. SK 33, Alcalase, pepsin and trypsin, respectively.

3.4.4 Antioxidant activity of FBS and RD hydrolysates

3.4.4.1 ABTS radical scavenging activity assay

FBS hydrolysates prepared from any proteinases showed higher ABTS radical scavenging activity than RD hydrolysates at 8 h hydrolysis ($p<0.05$, Figure 3.3). Antioxidant activity of hydrolysates greatly depends on their amino acid composition and specific amino acid sequence (Chen, Muramoto, Yamauchi, Fujimoto, and Nokihara, 1998). Several amino acids, such as Tyr, Met, His, Lys, and Trp, possessed antioxidant activity (Chen, Muramoto, Yamauchi, and Nokihara, 1996). Trp, Tyr and His residue contained the indolic, phenolic, and imidazole groups, respectively, which serve as hydrogen donors. FBS hydrolysates contained higher amounts of Lys, Tyr and His compared to RD hydrolysates (Table 3.4), which could partly contribute to higher ABTS radical scavenging activity observed in FBS hydrolysates.

FBS hydrolysate prepared by pepsin (5% DH) and *Virgibacillus* sp. SK33 proteinase (13% DH) showed high ABTS radical scavenging activity, while theABTS radical scavenging activity of the hydrolysates prepared by Alcalase (27% DH) and trypsin (11% DH) were comparable and lower than those prepared from pepsin ($p<0.05$). Our results indicated that antioxidant activity does not necessarily increase with DH. Specific amino acid composition in the peptide sequences is a more important factor manifesting antioxidant activity of protein hydrolysate. Jun, Park, Jung, and Kim (2004) also reported that the lowest DH of pepsin-hydrolyzed yellowfin sole frame exhibited higher antioxidant activity than those hydrolyzed by Alcalase and trypsin.

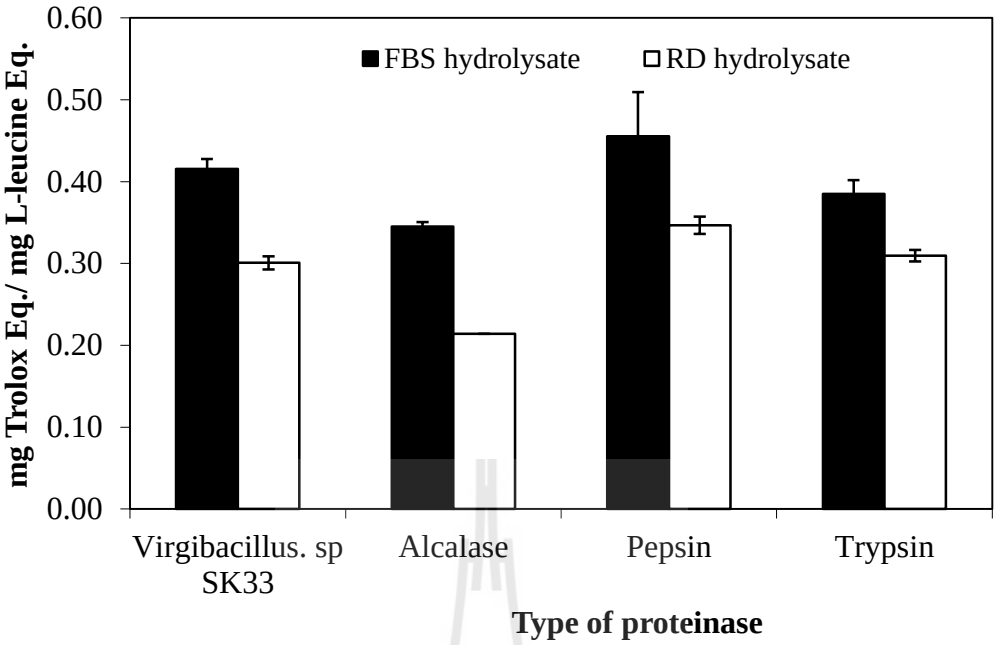

Figure 3.3 ABTS radical scavenging activity of FBS and RD hydrolysates treated with various enzymes for 8 h. Values are expressed as the mean ± S.D. (n=2).

3.4.4.2 Ferric reducing antioxidant power assay (FRAP assay)

FBS hydrolysates prepared from all tested proteinases showed higher reducing power than RD hydrolysates ($p<0.05$, Figure 3.4), which was in agreement with ABTS radical scavenging activity. The FBS hydrolysate prepared from pepsin at 5% DH showed the highest reducing power, followed by hydrolysates prepared from *Virgibacillus* sp. SK33 (13% DH) ~ trypsin (11% DH) and Alcalase (27% DH), respectively. A decrease in the reducing power as DH increased has been reported in Alcalase-hydrolyzed yellow stripe trevally (Klompong, Benjakul, Kantachote, and Shahidi, 2007) and peanut (Jamdar et al., 2010). This result confirms that the reducing power ability of protein hydrolysate vastly depends on the type of proteinase.

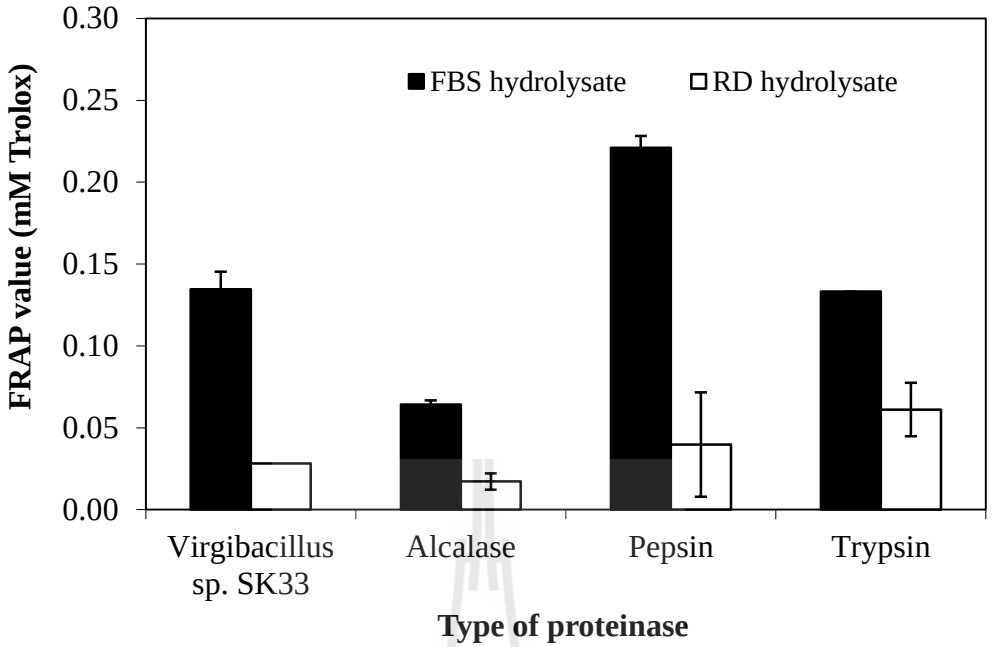

Figure 3.4 FRAP value of FBS and RD hydrolysates obtained from various enzymes for 8 h. Values are expressed as the mean ± S.D. (n=2).

3.4.4.3 Inhibition of β-carotene bleaching

All hydrolysates prevent β-carotene bleaching by donating hydrogen atoms to peroxyl radicals of linoleic acid ($p<0.05$, Figure 3.5). These results suggested that FBS and RD hydrolysates showed antioxidant activity not only in aqueous systems, but also in the emulsion model. This would imply the potential application of FBS and RD hydrolysates as antioxidants in food emulsion.

FBS hydrolysates showed higher β-carotene bleaching ability than RD hydrolysates regardless of the enzyme used ($p<0.05$, Figure 3.5). These results correlated well with ABTS radical scavenging and FRAP assays. Hydrolysates rich in peptides containing hydrophobic amino acids, such as Pro, Leu, Ala, Trp and Phe enhance antioxidant activity by increasing the solubility of peptides in lipid phase

(Mendis et al., 2005). In addition, His and Tyr could make reactive oxygen species (ROS) stable through electron/proton transfer (Qian et al., 2008). In our study, all FBS hydrolysates were richer in Tyr, His and hydrophobic amino acids including Val, Ile, Leu and Phe than RD hydrohysates (Table 3.4). This could contribute to a higher degree in the inhibition of β-carotene bleaching of FBS hydrolysates.

The pepsin-hydrolyzed FBS exhibited the highest β-carotene bleaching activity, while those prepared from Alcalase showed the lowest (Figure3.5). Besides acting as a radical scavenger and reducing power, peptide could serve as a protecting membrane surrounding lipid droplets against oxidation initiators (Hirose and Miyashita, 1999). Kong and Xiong (2006) reported that high DH of zein hydrolysates prepared by Alcalase reduced the peptide's ability to act as a physical barrier to prevent oxidants from reaching the lipid fraction in the liposome. This could explain the lowest inhibition of β-carotene bleaching of hydrolysates prepared by Alcalase. From these results, FBS hydrolysates prepared from pepsin were the most effective in hydrogen-donating ability.

3.4.5 Cytoprotective effect of FBS and RD hydrolysates

Cytotoxic of FBS and RD hydrolysates prepared from *Virgibacillus* sp. SK 33 proteinase was tested on HepG2 cell line to determine non-cytotoxic concentration for experiments of cytoprotective effect, and the results showed that both two hydrolysates shows any no cytotoxic effects on HepG2 cell line at peptide concentrations of 0-100 µg leucine equivalent/ml (Appendix A, Figure 1A). Therefore, it was determined that FBS and RD hydrolysates could be used for the investigation of the cytoprotective effect. When cells were treated with various concentrations of TBHP (0-1200 µM) for 2 h, they were killed in a dose-dependent

manner (Appendix A, Figure 2A). About 44% cell death was observed after cells were exposed to 500 µM TBHP. This concentration was, thus, chosen for the investigation of the protective effect of protein hydrolysates.

All hydrolysates at 12.5 µg leucine equivalents/ml showed a cytoprotective effect on TBHP in HepG2 cell (Figure 3.6). Surprisingly, FBS and RD hydrolysates showed a comparable cytoprotective effect ($p>0.05$), except for hydrolysates prepared from trypsin despite that FBS hydrolysates exhibited higher antioxidant activity based on all selected chemical assays (Figures 3.3-3.5). The differences in chemical and biological assays could be due to the type of radicals generated and the different protection mechanisms involved. $ABTS^{\bullet+}$ and Fe^{3+} were generated in ABTS radical scavenging and FRAP assays, respectively, while in the cell-based assay, numerous free radicals were generated, including $RO^{\bullet}$, $ROO^{\bullet}$, superoxide and hydroxyl radicals. Therefore, peptides could react with different radicals in different manners. In addition, most RD hydrolysates, except those prepared from *Virgibacillus* sp. SK33 proteinase and trypsin, contained higher amounts of total hydrophobic amino acids than did FBS hydrolysates (Table 3.4). Therefore, RD hydrolysates may efficiently penetrate into lipid bilayer of HepG2 cells and react with free radicals inside cells.

RD hydrolysates prepared from pepsin, trypsin and Alcalase showed comparable cytoprotective ability ($p>0.05$), while cell viability was lowest in samples treated with the hydrolysate prepared from *Virgibacillus* sp. SK33 and ascorbic acid ($p<0.05$) (Figure 3.6). Rajaparke et al. (2005) reported that purified peptide from giant squid muscle prepared from trypsin, NGLEGLK (747 Da), enhanced the cell viability of TBHP-induced cytotoxicity on embryonic lung fibroblasts (MRC-5) to a greater extent than did NADFGLNGLEGLA (1307 Da). The difference in the cytoprotective

ability of both peptides could be because lower molecular weight peptides effectively interacted with membrane lipids, resulting in higher cell permeability. This could explain the ability to protect cytotoxicity of hydrolysates prepared from Alcalase, which was expected to contain lower molecular weight peptides as it showed the highest DH. In addition, high antioxidant activity of His-containing peptides may be attributed to lipid radical-trapping ability of the imidazole ring, metal chelating, and hydroxyl radical scavenging (Chen et al., 1998). Kim, Je, and Kim (2007) reported that the purified peptide from hoki frame protein prepared from pepsin, ESTVPERTHPACPEPN prevented TBHP-induced cytotoxicity on MRC-5 cells by scavenging lipid-derived radicals. Most bioactive peptides contained His residues in their sequences. In our study, FBS and RD hydrolysates prepared from *Virgibacillus* sp. SK33 showed the lowest His compared to other hydrolysates (Table 3.4). This would result in the lowest cytoprotective ability on HepG2 cells. Furthermore, Minotti (1989) reported that TBHP could induce the release of iron following hydroperoxide metabolic activation by cytochrome P450 in hepatocytes. Carboxyl and amino groups at side chains of acidic (Glu, Asp) and basic (Lys, His, Arg) amino acids could act as a metal chelator. Such amino acids in hydrolysates prepared from *Virgibacillus* sp. SK33 were also the lowest (Table 3.4). This might also partly contribute to the lowest cytoprotective ability on HepG2 of the hydrolysate prepared from *Virgibacillus* sp. SK33.

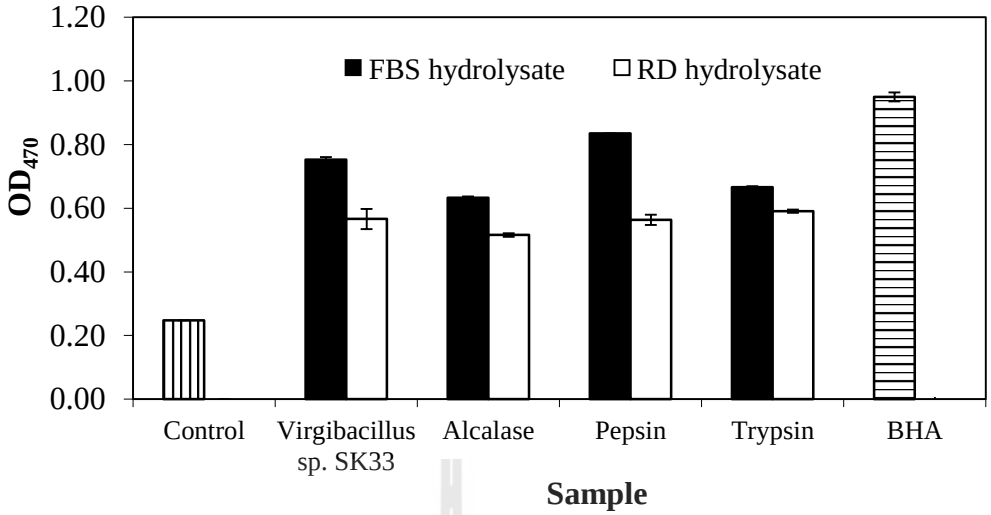

Figure 3.5 β-Carotene bleaching of FBS and RD hydrolysatestreated with various enzymes for 8 h. 0.01% BHA was used as a positive control. Values are expressed as the mean ± S.D. (n=2).

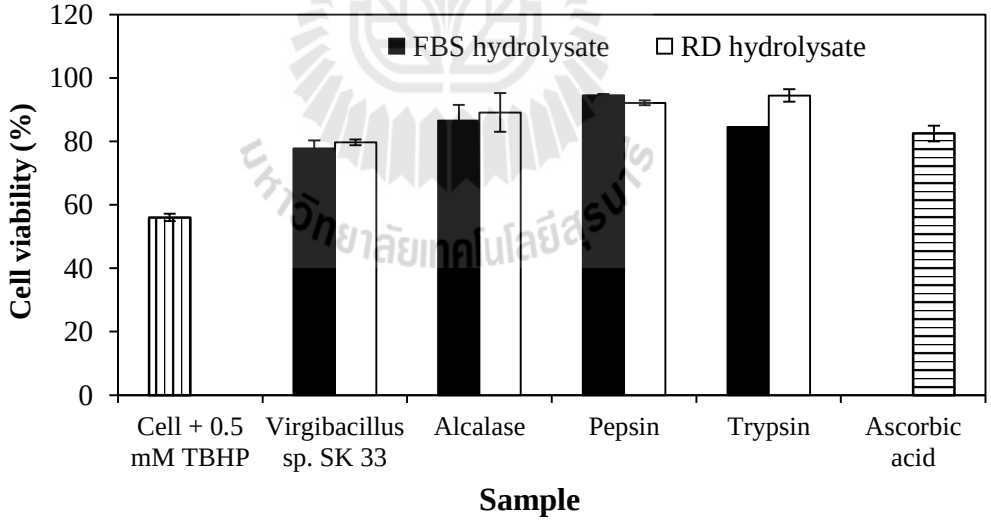

Figure 3.6 Effects of FBS and RD hydrolysates at 12.5 µg leucine equivalents/ml treated with various enzymes for 8 h on TBHP-induced cytotoxcixity to HepG2 cells. Ascorbic acid at 2.5µg/ml was used as positive control. Values are expressed as the mean ± S.D. (n=4).

3.5 Conclusions

ABTS radical scavenging, FRAP and β-carotene bleaching activities of FBS and RD protein hydrolysates showed a similar pattern, but did not correlate with cell-based assay. FBS hydrolysate prepared from pepsin showed the highest antioxidant activity based on chemical and biological assays. Therefore, pepsin-hydrolyzed FBS is a potential source of bioactive food ingredients.

3.6 References

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolyzates by trinitrobenzenesulfonic acid. **Journal of Agricultural and Food Chemistry.** 27: 1256-1262.

Alamed, J., Chaiyasit, W., McClements., D. J., and Decker, E. A. (2009). Relationships between free radical scavenging and antioxidant activity in foods. **Journal of Agricultural and Food Chemistry.** 57: 2969-2976.

AOAC (2000). Official methods of analysis (17th ed.). Gaithersberg, Maryland: Association of Official Chemists.

Arnao, M. B., Cano, A., and Acosta, M. (2001). The hydrophilic and lipophilic contribution to total antioxidant activity. **Food Chemistry.** 73: 239-244.

Benzie, I. F. F. and Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. **Analytical Biochemistry.** 239: 70-76.

Chen, H. M., Muramoto, K., Yamauchi, F., Fujimoto, K., and 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.

Chen, H. M., Muramoto, K., Yamauchi, F., and Nokihara, K. (1996). Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. **Journal of Agricultural and Food Chemistry.** 44: 2619-2623.

Dávalos, A., Miguel, M., Bartolomé, B., and López-Fandiño, R. (2004). Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. **Journal of food protection.** 67: 1939-1944.

Doucet, D., Otter, D. E., Gauthier, S. F., and Foegeding, E. A. (2003). Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: Peptide identification and determination of enzyme specificity. **Journal of Agricultural and Food Chemistry.** 50: 6300-6308.

FAO/WHO. (1975). Energy and protein requirements. PAG Bulletin, 5, 30-35 [PAG, New York].

FAO/WHO. (1991). Protein quality evaluation. Report of joint FAO/WHO expert consultation. FAO food and nutrition paper 51. Italy: Rome.

FAO/WHO/UNU (1985). Energy and protein requirement, WHO technical report series no. 724. Geneva, Switzerland: WHO.

Hirose, A. and Miyashita, K. (1999). Inhibitory effect of proteins and their hydrolysates on the oxidation of triacylglycerols containing docosahexaenoic acids in emulsion. **Journal of the Japanese Society for Food Science and Technology.** 46: 799-805.

Jamdar, S. N., Rajalakshmi, V., Pednekar, M. D., Juan, F., Yardi, V., and Sharma, A. (2010). Influence of degree of hydrolysis on functional properties, antioxidant

activity and ACE inhibitory activity of peanut protein hydrolysate. **Food Chemistry.** 121: 178-184.

Jun, S. Y., Park, P. J., Jung, W. K., and 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.

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

Kim, J. S. and Park, J. W. (2004). Characterization of acid-soluble collagen from Pacific whiting surimi processing byproducts. **Journal of Food Science.** 69:637-642.

Kim, J. S. and Park, J. W. (2005). Partially purified collagen from refiner discharge of Pacific Whiting surimi processing. **Journal of Food Science**. 70: c511-c516.

Klomklao, S., Kishimura, H., Yabe, M., and Benjakul, S. (2007). Purification and characterization of two pepsins from the stomach of pectoral rattail (*Coryphaenoides pectoralis*). **Comparative Biochemistry and Physiology, Part B**. 147: 682-689.

Klompong, V., Benjakul, S., Kantachote, D., and Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. **Food Chemistry**. 102: 1317-1327.

Koleva, I. I., van Beek, T. A., Linssen, J. P. H., de Groot, A., and Evstatieva, L. N. (2002). Screening of plant extracts for antioxidant activity: A comparative study

on three testing methods. **Phytochemical Analysis.** 13: 8-17.

Kong B. and Xiong, Y. L. (2006). Antioxidant activity of zein hydrolysates in a liposome system and the possible mode of action. **Journal of Agricultural and Food Chemistry.** 54: 6059-6068.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of head of bacteriophage T4. **Nature.** 227: 680-685.

Lu, B. J., Zhou, L. G., and Cai, Q. F. (2008). Purification and characterisation of trypsins from the pyloric caeca of mandarin fish (*Siniperca chuatsi*). **Food Chemistry.** 110: 352-360.

Mendis, E., Rajapakse, N., and Kim, S. K. (2005). Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. **Journal of Agricultural and Food Chemistry.** 53: 581-587.

Minotti, G. (1989). *tert*-Butylhydroperoxide-dependent microsomal release of iron and lipid peroxidation: I. Evidence for the reductive release of nonheme, nonferritin iron. **Archives of Biochemistry and Biophysics.** 273: 137-143.

Morrisey, M. T., Lin, J., and Ismond, A. (2005). Waste management and waste utilization. In J. W. Park (Ed.). **Surimi and surimi seafood** (second ed., pp. 279-323). New York: Marcel Dekker.

Narayanan, V. S., Fitch, C. A., and Levenson, C. W. (2001). Tumor suppressor protein p53 mRNA and subcellular localization are altered by changes in cellular copper in human HepG2 cells. **Journal of Nutrition.** 131: 1427-1432.

Oluwaniyi, O. O., Dosumu, O. O., and Awolola, G. V. (2010). Effect of local processing methods (boiling, frying and roasting) on the amino acid composition of four marine fishes commonly consumed in Nigeria. **Food**

Chemistry. 123: 1000-1006.

Park, P. J., Jung, W. K., Nam, K. S., Shahidi, F., and Kim, S. K. (2001). Purification and characterization of antioxidative peptides from protein hydrolysate of lecithin-free egg yolk. **Journal of the American Oil Chemists Society**. 78: 651-656.

Pangsorn, S., Laong-manee, P., and Siriraksophon, S. (2007). Status of Surimi Industry in the Southeast Asia. TD/RES 118, SEAFDEC, Training Department, Samutprakan, Thailand; 3, 6 pp

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

Rajapakse, N., Mendis, E., Byun, H. G., and 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.

Simpson, B. K. (2000). Digestive proteinases from marine animals. In N. M., Haard, and B. K. Simpson (Eds.). **Seafood enzymes: Utilization and influence on postharvest seafood quality** (pp. 531-540). New York: Marcel Dekker.

Smith, C. A., Toogood, H. S., Baker, H. M., Daniel, R. M., and Baker, E. N. (1999). Calcium-mediated thermostability in the subtilisin superfamily: The crystal structure of Bacillus Ak.1 protease at 1.8 Å resolution. **Journal of Molecular Biology.** 294: 1027-1040.

Sinsuwan, S., Rodtong, S., and Yongsawatdigul, J. (2008). Production and characterization of NaCl-activated proteinases from *Virgibacillus* sp. SK33 isolated from fish sauce fermentation. **Process Biochemistry**. 43: 185-192.

Sinsuwan, S., Rodtong, S., and Yongsawatdigul, J. (2010). A NaCl-stable serine proteinase from *Virgibacillus* sp. SK33 isolated from Thai fish sauce. **Food Chemistry.** 119: 573-579.

Suárez, M. D., Martínez, T. F., Sáez, M. I., Morales, A. E., and García-Gallego, M. (2010). Effects of dietary restriction on post-mortem changes in white muscle of sea bream (*Sparus aurata*). **Aquaculture.** 307: 49-55.

CHAPTER IV

ISOLATION AND IDENTIFICATION OF ANTIOXIDATIVE PEPTIDES FROM HYDROLYSATE OF THREADFIN BREAM SURIMI PROCESSING BYPRODUCT

4.1 Abstract

The objective of this study was to determine the antioxidant activity and possible mode of action of partially purified peptides derived from threadfin bream surimi byproduct. The frame, bone and skin (FBS) byproduct, which was obtained from a deboning process, was hydrolyzed by *Virgibacillus* sp. SK33 proteinase and fractionated using anion exchange and size exclusion chromatography. Three fractions, i.e., B1, B2 and B3, were obtained, and the amino acid sequences of the peptides in all 3 fractions were determined using LC-MS/MS. Fractions B2 and B3 contained higher amounts of Trp, Met, Cys and Tyr residues than fraction B1. Fraction B3 exhibited high ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP), while metal chelation and hydroxyl radical scavenging ability were principle modes of action of peptides in fraction B2 and B3. In addition, fraction B1 and a synthetic peptide selected from the pooled *de novo* peptides of fraction B3, FLGSFLYEYSR, had a cellular radical scavenging effect when HepG2 cells were treated with H_2O_2.

Keywords: Antioxidant, peptides, mode of action, threadfin bream, surimi byproducts

4.2 Introduction

In aerobic organisms, reactive oxygen species (ROS), such as superoxide radical ($O_2^{-\bullet}$), hydroxyl radical ($HO^{\bullet}$), and hydrogen peroxide (H_2O_2), are produced during normal physiological processes. These radicals are very unstable and rapidly react with other groups or substances in the body, leading to cell or tissue injury (Ames, Shigena, and Hagen, 1993). In addition, metal ions are known to induce free radical formation through a variety of processes, including the Fenton reaction (Halliwell, Aeschbach, Loliger, and Aruoma, 1995). Under normal conditions, cells are protected against oxidation by both enzymatic and non-enzymatic antioxidant pathways. However, excessive ROS can damage DNA, proteins, and lipids, thereby contributing to many serious diseases, such as liver disease and cancer (Leanderson, Faresjo, and Tagesson, 1997; Vitaglione, Morisco, Caporaso, and Fogliano, 2004). Therefore, natural antioxidants that enhance antioxidant defenses in the body have been continually sought.

Some food protein hydrolysates have been reported to exhibit antioxidant activity, such as porcine myofibrillar protein (Saiga, Tanabe, and Nishimura, 2003), porcine collagen (Li, Chen, Wang, Ji, and Wu, 2007), tuna backbone (Je, Qian, Byun, and Kim, 2007), hoki frame (Kim, Je, and Kim, 2007), potato (Kudo, Onodera, Takeda, Benkebia, and Shiomi, 2009), royal jelly protein from the honeybee (Guo, Kouzuma, and Yonekura, 2009), Nile tilapia scale (Ngo, Qian, Ryu, Park, and Kim, 2010), rapeseed (He, Girgih, Malomo, Ju, and Aluko, 2013), and tilapia (Sun, Zhang, and Zhuang, 2013). These hydrolysates were composed of 2-16 amino acids, and their

activities depended on size, amino acid composition, and sequence. These hydrolysates exhibit antioxidant activity bydifferent modes of action: the donation of electrons/hydrogen atoms, direct scavenging of free radicals, and sequestration of pro-oxidative metal ions.

Our previous study revealed that an FBS hydrolysate with antioxidant activity can be obtained with *Virgibacillus* sp. SK33 proteinase (Wiriyaphan, Chitsomboon, and Yongsawadigul, 2012). However, the antioxidant mode of action of the peptides in the FBS hydrolysate is still unknown. Therefore, the objectives of this study were to fractionate and identify the antioxidant peptides from an FBS hydrolysate prepared from *Virgibacillus* sp. SK33 proteinase and to elucidate the possible mode of action of the antioxidant peptides in both cellular and non-cellular oxidative systems.

4.3 Materials and methods

4.3.1 Materials

The mixture of frame, bone, and skin (FBS), which was obtained from a deboning process of threadfin bream surimi production, was donated by the Andaman Surimi Industry (Samutsakorn, Thailand). FBS was packed in a polystyrene foam box filled with ice and transported to a laboratory at the Suranaree University of Technology. FBS was dried, ground, and defatted as described in Chapter III (section 3.3.1). Defatted FBS powder contained 41.70±2.68% crude protein and 47.90±1.86% ash on a dry matter basis. *Virgibacillus* sp. SK 33 isolated from one-month-old Thai fish sauce mashes was used as the source of proteinase. Details of *Virgibacillus* sp. SK33 proteinase production were described in Chapter III (section 3.3.2). *Virgibacillus* sp. SK33 proteinase activity was assayed following the method of

Sinsuwan, Rodtong, and Yongsawatdigul (2008) using Suc-Ala-Ala-Pro-Phe-aminomethylcoumarin (AMC) as substrate, at 50 °C and pH 8. One unit is the amount of enzyme that releases 1 nmole of AMC per min. The compounds 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were purchased from BioChemika (Buchs, Switzerland). The compounds 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), xanthine, 2-deoxy-D-ribose, and xanthine oxidase from bovinemilk were purchased fromSigma Chemical Co. (St. Louis, MO, USA).The HepG2 human hepatoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle Medium, Nutrient Mixture F-12 (DMEM-F12), was purchased from Gibco BRL, Life Technologies (Gaithersburg, MD, USA). The other chemicals and reagents used were of analytical grade.

4.3.2 Production of a defatted FBS hydrolysate

Ten grams of defatted FBS powder were added 0.1 M phosphate buffer at pH 8.0 (10%, w/v) and hydrolyzed with *Virgibacillus* sp. SK33 proteinase at 13.5 U/g powder at 65°C, which was found to be the optimal temperature for FBS hydrolysis, for 8 h. The hydrolysate was heated at 90 °C for 10 min to stop the reaction and centrifuged at 10,000×g for 20 min at 4 °C. The supernatants were lyophilized and stored at -20 °C until further use. The peptide content was determined using the Lowry method with tyrosine as a standard. Degree of hydrolysis was determined as described in Chapter III (section 3.3.5.1).

4.3.3 Purification of the antioxidant peptides from the FBS hydrolysate

4.3.3.1 Ion exchange chromatography

The lyophilized FBS hydrolysate (0.33 g) was dissolved in deionized

water (8 ml). One ml of peptide solution was applied onto a DEAE–Sephacel ion exchange column (2.6 x 6.5 cm, GE Healthcare, Piscataway, NJ, USA) equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The elution was performed using an AKTA Purifier (GE Healthcare, Piscataway, NJ, USA) with a linear gradient of NaCl (0–1.0 M) at a flow rate of 1 ml/min. The eluate was monitored at 215 nm and collected in 5 ml-volume fractions. Ion exchange chromatography was repeated 5 times with a total loading volume of 5 ml. Fractions with high absorbance at 215 nm from each injection were pooled and lyophilized. The peptide content was determined using the Lowry method with tyrosine as a standard (Lowry, Rosebrough, Farr, and Randall, 1951). The antioxidant activity of each pooled fraction was measured using the ABTS radical scavenging activity and FRAP assays as described in Chapter III (sections 3.3.5.2.1 and 3.3.5.2.2, respectively) at peptide concentration of 0.1 mg tyrosine equivalents/ml. The pooled fraction with the highest antioxidant activity was used for further purification.

4.3.3.2 Size exclusion chromatography (SEC)

The lyophilized powder obtained from DEAE-Sephacel ion exchange chromatography (1.2 g) was dissolved in deionized water (15 ml). Three ml of reconstituted peptide was applied onto a Superdex 30 prep grade column (1.6 x 100 cm, GE Healthcare, Piscataway, NJ, USA) equilibrated with deionized water. The elution was performed using an AKTA Purifier (GE Healthcare, Piscataway, NJ, USA) with an isocratic mode with deionized water at a flow rate of 0.5 ml/min. The eluate was monitored at 215 nm and collected in 3 ml-volume fractions. The purification was repeated 5 times with the total loading volume of 15 ml. The pooled fractions were then lyophilized. The peptide content was determined using the Lowry

method with tyrosine as a standard (Lowry et al., 1951). The antioxidant activity of each lyophilized fraction at varied concentrations was measured following 4 assays as described in section 4.3.6.

4.3.4 LC-MS/MS

The amino acid sequences of the fractionated peptides were determined using an Ultimate 3000 LC System (Dionex, Bannockburn, IL) coupled to an ESI-Ion Trap MS (HCT Ultra PTM Discovery System, Bruker, Daltonics, Germany) with electrospray ionization. The peptides were separated in a nanocolumn (Acclaim PepMap 100 C18, 3 mm, 100A, 75 mm id x 150 mm). Eluent A consisted of 0.1% formic acid in water, and eluent B consisted of 80% acetonitrile in water with 0.1% formic acid. The elution was performed using a 13-min linear gradient of 0 to 70% of eluent B at a flow rate of 300 nl/min. De novo sequencing was carried out with Pepnovo and can be accessed through http://proteomics.ucsd.edu/LiveSearch/.

4.3.5 Peptide synthesis

Since fraction B3 obtained from size exclusion chromatography exhibited high antioxidant activity at various modes with the highest peptide yield, antioxidant peptides contained in this fraction were sought. Peptides of fraction B3 whose sequences were obtained from LC-MS/MS were synthesized using a solid phase peptide synthesis method (GL Biochem, Shanghai, Ltd.). The synthetic peptides, including VELLVPK, LGTGTDL and NTFLFFK, were purified using an HPLC in a Amethyst C18-H (4.6 × 250 mm) column; AGNQVLNLQADLPK and FLGSFLYEYSR were purified in a Crest ODS (4.6 × 250 mm) and SinoChrom ODS-BP (4.6 × 250 mm) column, respectively. The molecular mass of the synthetic peptides was determined by chromatography coupled to a mass spectrometry (LC–

MS/ESI). The peptide NTFLFFK was only soluble in trifluoroacetic acid which interfered with most antioxidant assays except for ABTS radical scavenging activity. Thus, this peptide was eliminated from the study. Therefore, antioxidant activity of 4 peptides at various modes of action was individually determined at 5 mg/ml. Three peptides, namely VELLVPK, LGTGTDL, and AGNQVLNLQADLPK, were dissolved in deionized water, while FLGSFLYEYSR peptide was dissolved in dimethyl sulfoxide (DMSO), for antioxidant activity assays. The mixtures of these peptides at equal concentration to attain final concentration of 5 mg/ml were also determined for their antioxidant activity at various modes.

4.3.6 Mode of action of the purified peptides

4.3.6.1 ABTS radical scavenging activity assay

The fractionated peptides (B1, B2 and B3) after SEC were dissolved in deionized water at various concentrations. The ABTS assay was carried out as described in Chapter III (section 3.3.5.2.1). Results were expressed as mM Trolox equivalents.

4.3.6.2 FRAP assay

The fractionated peptides (B1, B2 and B3) after SEC were dissolved in deionized water at various concentrations. The FRAP assay was carried out as described in Chapter III (section 3.3.5.2.2). Results were expressed as µM Trolox equivalents.

4.3.6.3 Metal chelating assay

The ability of peptides to chelate to ferrous ions (Fe^{2+}) was assessed using the method reported by Decker and Welch (1990) with some modifications. The fractionated peptides (B1, B2 and B3) were dissolved in deionized water at various

concentrations. One hundred µl of peptide solution was mixed with 2400 µl of deionized water, 50 µl of 2 mM $FeCl_2$ and 100 µl of 5 mM 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine). The mixture was incubated at room temperature for 20 min in the dark. The color of the ferrous iron–ferrozine complex was measured at 562 nm. Results were expressed as mM Ethylenediaminetetraacetic acid (EDTA) equivalents.

4.3.6.4 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was assessed using the 2-deoxyribose oxidation method according to Sakanaka and Tachibana (2006) with some modifications. Hydroxyl radicals were generated by a Fenton reaction. Each fractionated peptide was dissolved in deionized water at various concentrations. One hundred µl of peptide solution was mixed with 0.45 ml of 0.2 M sodium phosphate buffer (pH 7.4), 0.15 ml of 10 mM 2-deoxyribose, 0.15 ml of 10 mM $FeSO_4$–EDTA, 0.15 ml of 10 mM hydrogen peroxide (H_2O_2), and 0.5 ml of deionized water. The mixture was incubated for 4 h in a 37°C-water bath. Subsequently, 0.75 ml of 2.8% trichloroacetic acid and 0.75 ml of 1.0% thiobarbituric acid were added. The reaction mixture was then boiled for 10 min and cooled in ice; the absorbance was measured at 520 nm. A control was prepared using 100 µl of deionized water instead of the peptide solution. The hydroxyl radical scavenging activity was calculated as percentage of inhibition using the following equation (Sakanaka and Tachibana, 2006):

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{\text{Absorbance of control}}$$

4.3.7 Cell culture and Cellular ROS determination by DCFH-DA

HepG2 cells were cultured in DMEM-F12 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. After the cells had reached 90% confluency, they were sub-cultured by trypsinization. Intracellular formation of ROS was assessed using oxidation sensitive dye 2′, 7′-dichlorofluorescin-diacetate (DCFH-DA) as a substrate according to Qian, Jung, Byun, and Kim (2008) with some modifications. Cells were plated in fluorescent microtiter 96-well plates (3.0×10^4 cells per well) and incubated for 24 h. Subsequently, the cells were pre-treated with either the fractionated peptide (B1) or a synthetic peptide (FLGSFLYEYSR) at a final concentration of 10 and 20 µg/ml for 1 h. The culture medium was removed, and 10 µM DCFH-DA in Hank's balanced salt solution (HBSS) was added to the cells, which were then incubated in the dark for 30 min. The cells were washed three times in PBS. Subsequently, 0.3 mM H_2O_2 in HBSS was added to the cells, which were incubated for 1 h. The DCFH was oxidized by the ROS resulting in the formation of 2', 7'-dichlorofluorescin (DCF), a fluorescent product, which was monitored at an excitation wavelength (E_x) of 485 nm and an emission wavelength (E_m) of 530 nm using a Spectramax Gemini EM fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA).

4.3.8 Statistical analyses

Experiments were repeated twice using 2 different lots of FBS. All tests were performed in duplicate. Statistical analyses were performed using the SPSS package (SPSS 17.0 for window, SPSS Inc, Chicago, IL, USA). Data were statistically analyzed by one-way analysis of variance (ANOVA). The comparison among the

means was conducted using Duncan's multiplerange test (DMRT) at $p < 0.05$.

4.4 Results and discussion

4.4.1 Purification of antioxidant peptides

The degree of hydrolysis of FBS by *Virgibacillus* sp. SK33 was 12.84%. The amount of tyrosine equivalent was used for peptide yield calculation. Crude hydrolysate containing approximately 188.6 mg tyrosine equivalents/g dry weight and a total of 39.3 mg tyrosine equivalents (0.21 g crude hydrolysate) was applied to anion exchange chromatography. Subsequently, both unbound (Fraction A) and bound (Fraction B) fraction were obtained (Figure 4.1a) with approximately 30% yield (Table 4.1). ABTS radical scavenging activity and FRAP value of the bound fraction were the highest (Table 4.1). Metal chelating ability and hydroxyl radical scavenging activity were also determined, but Tris-HCl buffer used in anion exchange chromatography interfered with such assays. Tris can interact with hydroxyl radicals ($^{\bullet}OH$), resulting in the formation of formaldehyde via the Fenton reaction (Shiraishi, Kataoka, Morita, and Umemoto, 1993). In addition, the amino groups of Tris contribute to the chelation of metal ions.

The DEAE-bound fraction was further purified using size-exclusion chromatography, yielding three fractions, namely B1, B2, and B3 (Figure 4.1b). Fraction B3 showed the highest yield of 50%, indicating that it was a major constituent of the bound fraction-B (Table 4.2). This fraction also exhibited the highest ABTS radical scavenging activity, and FRAP value, while fraction B2 showed the highest metal chelating activity and hydroxyl radical scavenging activity (Table 4.2, $p<0.05$). Fractions B2 and B3 were expected to contain smaller peptides than B1.

Our results suggested that smaller peptides exhibited higher antioxidant activity than did larger counterparts.

4.4.2 Mode of action of fractionated peptides

A total of 361 peptide fragments derived from FBS were obtained from *de novo* peptide sequencing: 282 peptides from B1, 74 peptides from B2, and 5 peptides from B3. Representative sequences from each fraction are shown in Table 4.3. Leu (L) appeared to be major amino acid in all 3 fractions, while Glu (E) was predominant in both B1 and B2 fractions. Basic amino acids, namely Lys (K) and Arg (R), were present in all 3 fractions. Trp (W) and Tyr (Y) were observed in fraction B1 and B2. Fraction B3 contained less Glu, but higher proportion of Tyr than others. These amino acids have been reported to contribute to antioxidant activity of peptides. Trp and Tyr contain the indole and phenolic groups, respectively, which serve as hydrogen donors (Hernández-Ledesma, Davalos, Bartolome, and Amigo, 2005). Glu, Asp, and Lys may interact with metal ions through their charged residues and inactivate oxidant activity of metal ions (Saiga et al., 2003). In addition, Arg was also presumed to have the ion chelation activity (Zhang et al., 2010).

Antioxidant activity of all 3 fractions as a function of concentration (tyrosine equivalent) was investigated. Due to relatively low antioxidant activity of fraction B1, its peptide concentration was set in the range of 0.5-2.5 mg/ml. The highest concentration remained completely soluble in water of fraction B2 and B3 peptides was at 0.05 and 0.15 mg/ml, respectively, which was, therefore, set as the maximum concentration for antioxidant activity assays. Low solubility of fraction B2 was in agreement with high proportion of hydrophobic amino acids, i.e. Leu, Phe, and Trp, contained in these peptides.

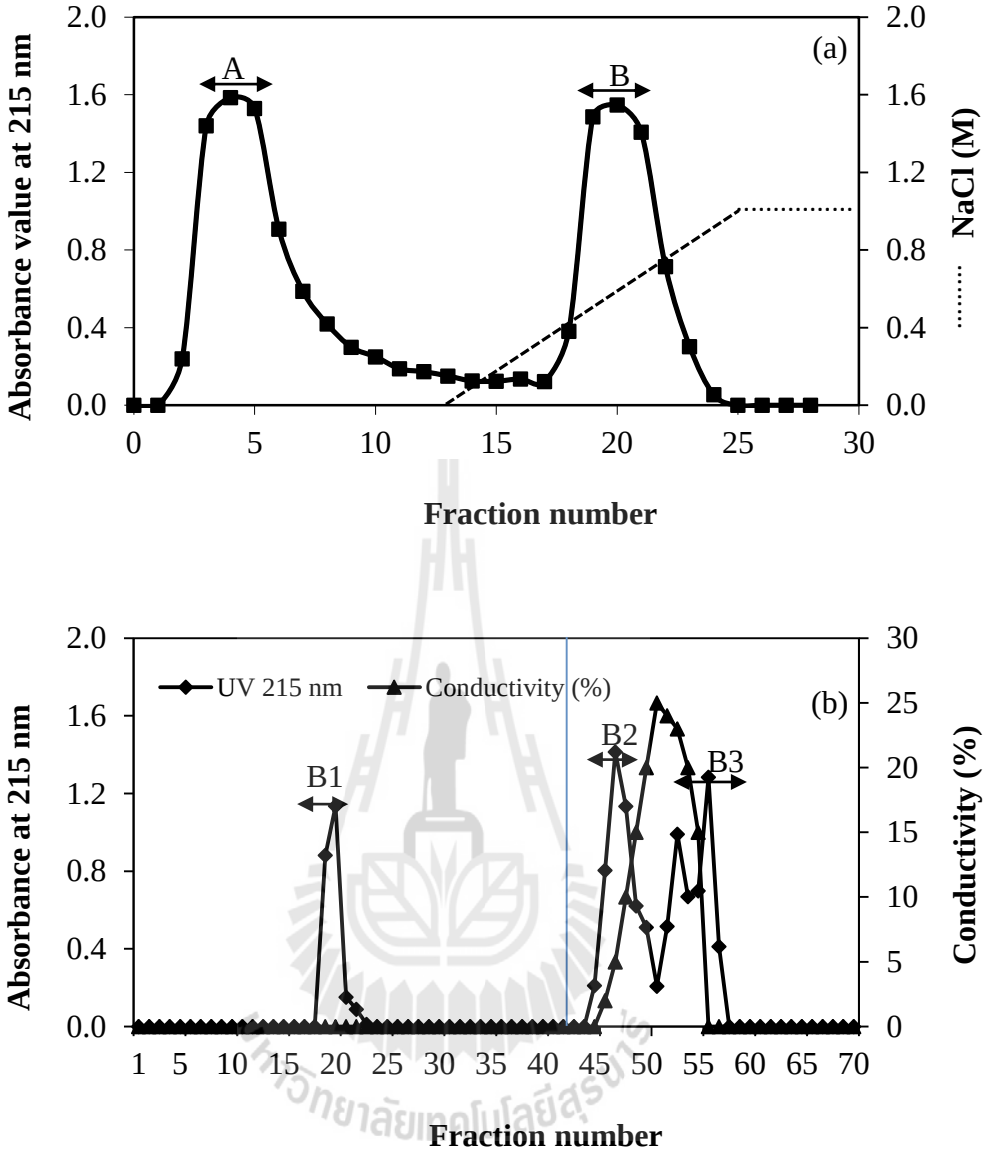

Figure 4.1 The purification of the antioxidant peptides from an FBS hydrolysate prepared from *Virgibacillus* sp. SK33 proteinase. (a) DEAE-Sephacel chromatogram. Elution was performed at a 1 ml/min-flow rate with a linear gradient of NaCl (0–1 M) in 50 mMTris-HCl buffer at pH 8.0. (b) Chromatogram of the active fraction B from Figure 1a using Superdex 30 prep size exclusion chromatography. Elution was achieved at a 0.5 ml/min- flow rate with deionized water.

Table 4.1 Antioxidant activity of crude and DEAE-Sephacel fractions

Sample	ABTS radical scavenging activity (mM Trolox)	FRAP value (µM Trolox)	Peptide content (mg tyrosine equivalent/ g dry weight)	Total peptide (mg tyrosine equivalent)	Yield (%)
Crude hydrolysate	0.214 ± 0.002^{a}	14.993 ± 0.324^{b}	188.64	39.25	100
Unbound fraction-A	0.329 ± 0.002^{b}	5.926 ± 0.06^{a}	4.9	11.9	30
Bound fraction-B	0.617 ± 0.008^{c}	37.447 ± 4.377^{c}	1.7	12.0	31

Note: Antioxidant activities were tested at a concentration of 0.10 mg tyrosine equivalent/ml.

Different superscripts within a column indicate significant differences ($P < 0.05$).

Yield was calculated based on total mg tyrosine equivalent.

Table 4.2 Antioxidant activities and peptide yield obtained from size exclusion chromatography.

Fraction	ABTS radical scavenging activity (mMTrolox)	FRAP value (µM Trolox)	Metal chelating activity (mM EDTA)	Hydroxyl radical scavenging activity (%)	Peptide content (mg tyrosine equivalent/ g dry weight)	Total peptide (mg tyrosine equivalent)	Yield (%)
B1	0.090 ± 0.006^{a}	9.255 ± 0.113^{a}	0.185 ± 0.007^{a}	0^{a}	1.29	0.129	6
B2	0.154 ± 0.004^{a}	7.536 ± 1.897^{a}	0.687 ± 0.032^{c}	77.2 ± 0.8^{c}	0.12	0.262	13
B3	0.487 ± 0.061^{b}	113.398 ± 7.051^{b}	0.307 ± 0.051^{b}	55.3 ± 5.2^{b}	0.51	1.020	50

Note: Antioxidant activities were tested at 0.05 mg tyrosine equivalent/ml.

Different superscripts within a column indicate significant differences ($P < 0.05$).

Yield (%) was calculated from the initial content of 2.04 mg tyrosine equivalent of the bound fraction-B

Table 4.3 Peptides identified by LC coupled to ESI-Ion Trap MS in fractions B1, B2, and B3, after SEC.

Fraction	No.	Amino acid sequence	Fraction	No.	Amino acid sequence	Fraction	No.	Amino acid sequence
B1	1	L**DDE**FP**D**LSL**H**	**B2**	1	ALG**D**Q**R**TFFLNS**M**VL**EEHC**	**B3**	1	V**E**LLVP**K**
	2	V**EDE**FP**D**LSL**H**		2	LAG**D**Q**R**TFFLSN**M**VL**EEHC**		2	AGNQVLNLQA**D**LP**K**
	3	FSL**DDE**FP**D**LSL**H**		3	L**EDEEH**PSGAELT		3	LGTGT**D**L
	4	L**DEE**STLAGA**HE**		4	**E**L**E**ELAQGPV**Y**QA		4	FLGSFL**YEYS**R
	5	V**EEE**STLAGA**HE**		5	PT**RRYY**TLFVFL**KR**LLLGLFR**L**LLF**Y**A**Y**K**G**M**P**		5	NTFLFF**K**
	6	**H**ELFAAS**R**TLFLN**R**L**DD**LG**H**		6	AVL**WCQR**GFL**EEDE**L**K**LF			
	7	L**R**FSL**EHC**L**R**		7	**E**L**E**L**EEE**L**E**AQL**W**			
	8	FGL**EFHC**L**K**LF		8	GN**M**N**ME**K**L**W			
	9	GFL**EFCH**L**K**LF		9	L**EE**L**EEE**L**E**G**W**L			
	10	LQLV**EEWHK**N**W**VF		10	L**EE**L**EEE**L**E**GL**W**			
	11	ASL**EEAE**GTL**EHEEAK**LL**R**		11	N**C**AQ**ME**K**L**W			
	12	TL**DD**VLQT**RD**AL**C**P**H**Q		12	**WNDDM**TR**L**W			
	13	TGANP**D**LT**E**QL**W**TG**K**SL**E**L**HE**		13	ASL**EEAE**LL**D**QFF**C**LQL			
	14	**E**LFNLG**EAED**LGG**RY**LA		14	GLL**DE**L**FC**LGL**EEEE**L**K**LF			
	15	FSG**D**QL**EDY**TTTTV		15	LGL**DE**L**FC**LGL**EEEE**L**K**LF			
	16	FSG**D**QL**EDY**VA**W**F		16	VAL**DE**L**FC**LGL**EEEE**L**K**LF			
	17	KT**RW**N**EDE**L**Y**ALQ**KK**		17	FGL**EEEE**LFL**K**			
	18	L**E**QV**WY**LFNQSR**Y**L**E**		18	ALG**D**Q**R**TFFLNS**M**VL**EEHC**			
	19	LT**Y**NQ**D**LA**E**PR**C**FT**M**		19	LAG**D**Q**R**TFFLSN**M**VL**EEHC**			
	20	L**ED**SL**R**T**DE**L**Y**AQ		20	L**E**D**EEH**PSGAELT			
	21	LTRS**DLEDE**L**Y**AQL**K**		21	**E**L**EE**LAQGPV**Y**QA			
	22	NP***E***L***EE***LSFL**Y**AQ						
	23	NP**E**L**EE**LSFL**Y**AAG						
	24	NP**Y**V**ME**FNL**DKY**F**E**						
	25	PN**Y**V**ME**FNL**D**QL**YY**						
	26	QVQQ**E**L**Y**Q**W**R**F**EL**EM**L**R**QG**M**V						
	27	SAL**EEAE**GTLVGLF**RY**Q**E**L						
	28	TLN**Y**Q**D**LA**E**P**RC**FT**M**						
	29	TL**RD**SL**EDE**L**Y**AQ						
	30	**EE**L**R**AL**EEE**L**EA**W**QL						
	31	**ERD**LL**DR**W**L**FL**E**V**WME**L**E**						
	32	**EE**LA**R**L**EEE**L**EA**W**QL						
	33	GV**D**NLVFL**EWMD**LL**E**						
	34	L**EE**L**EEE**L**E**AL**W**Q						
	35	**R**W**N**W**RE**L**R**FL**EE**V**WM**L**E**						
	36	A**ER**AAFL**EEE**L**EKMR**F						
	37	FSG**D**QL**ED**LAG**M**FF						
	38	GA**E**QL**DM**TTS**D**L**E**LPPGT						
	39	**K**LED**M**QL**E**A**E**GG**C**K**LE**						
	40	LV**EEE**L**D**VGAQ**K**L**M**P						
	41	A**R**L**EE**L**EEE**L**EA**CK**PV						
	42	**E**L**EEE**L**EAK**PV**C**						
	43	L**EE**L**EEE**LA**KC**PV**E**						

Note: Amino acids reported to have antioxidant activity are shaded in grey and those reported to exhibit metal chelating activity are present in bold.

The ABTS antioxidant activity assay is based on the ability to quench the long-lived $ABTS^{\bullet+}$ using donated hydrogen atoms or electrons (Raghavan, Kristinsson, and Leeuwenburgh, 2008). Fractions B1 and B3 exhibited a dose-dependent ABTS radical scavenging activity at concentrations up to 2.5 and 0.15 mg/ml, respectively (Figures 4.2 a-b). The maximum solubility of fraction B2 was obtained at 0.05 mg/ml with ABTS radical scavenging activity of 0.154 mM Trolox (Table 4.2). Relatively low antioxidant activity of fraction B2 was mainly due to its low solubility (0.05 mg/ml). Antioxidant activity of fraction B1 could be attributed to Tyr, Trp, Cys, and His residues (Table 4.3), which have been reported to display ABTS radical scavenging activity (Hernández-Ledesma, Hsieh, and de Lumen, 2009; Sadat et al., 2011; Saiga et al., 2003).

The FRAP value of fractions B1 and B2 was about 15 times less than that of fraction B3 (Table 4.2). The FRAP value of fraction B1 and B3 increased almost linearly with peptide concentration (Figures 3a-b), while that of fraction B2 could not be determined at concentration > 0.05 mg/ml due to its low solubility. Two Tyr residues in one of peptides in fraction B3 (Table 4.3) could contribute to its highest FRAP value. It should be mentioned that fractions B1 and B2 displayed low FRAP value despite the presence of antioxidant amino acids (Table 4.3). This emphasizes the importance of amino sequence and size of peptides, which greatly influence antioxidant activity of peptides.

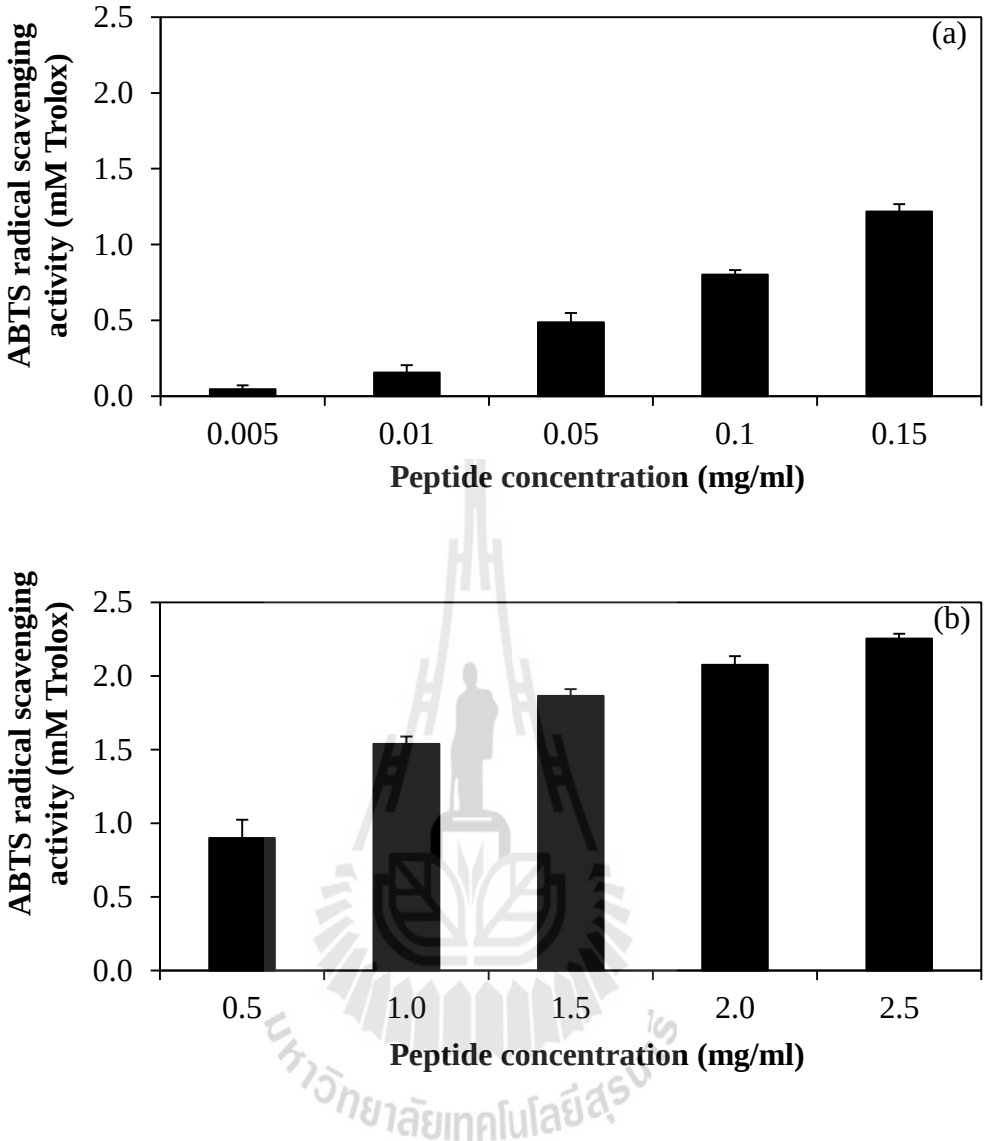

Figure 4.2 The effect of the peptide concentration on the ABTS radical scavenging activity of partially-purified peptides, fractions B1 (a), and B3 (b) obtained by chromatography using the Superdex 30 prep grade gel. Values are expressed as the mean ± S.D. (n=2).

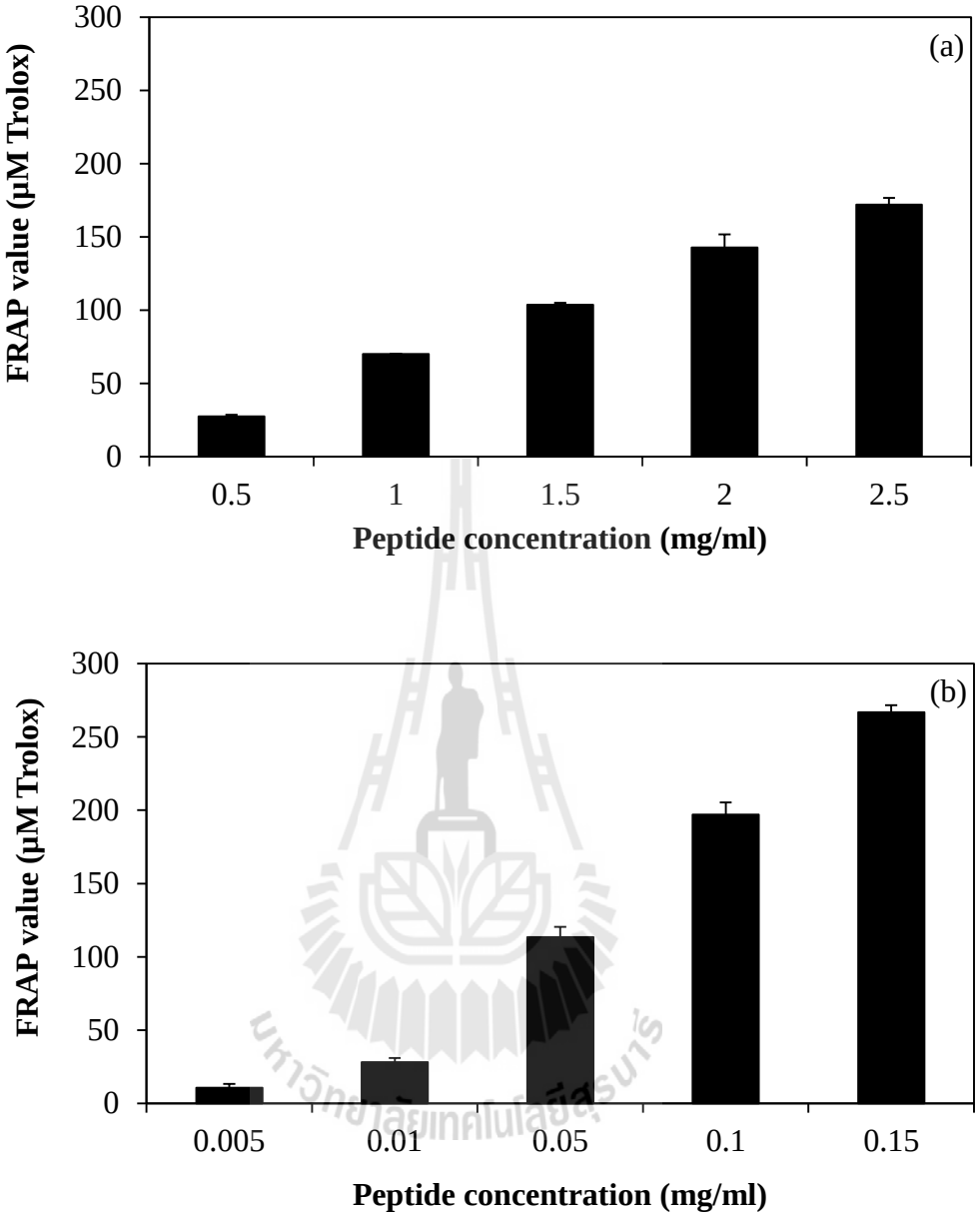

Figure 4.3 The effect of the peptide concentration on the FRAP value of partially-purified peptides , fractions B1 (a), and B3 (b) obtained by chromatography using the Superdex 30 prep grade gel. Values are expressed as the mean ± S.D. (n=2).

Transition metal ions, such as Fe^{2+} and Cu^{2+}, are involved in many oxidative reactions *in vivo*. They can catalyze the generation of hydroxyl and superoxide radicals that further oxidize unsaturated lipids (Stohs and Bagchi, 1995). The metal ion chelating ability of fraction B1 increased drastically at 0.1-0.3 mg/ml and remained constant at higher concentrations (Figure 4.4a). Effective metal chelating activity was observed in fraction B2 at concentration as low as 0.01 mg/ml (Figure 4b). It could be speculated that the prevalence of Glu could partly contribute to high metal chelating activity of fraction B2. The metal ion chelating activity of fraction B3 also increased with peptide concentration (Figure 4.4c). Acidic (Asp and Glu) and basic (His, Lys and Arg) amino acids with carboxyl and amino groups in their side chains contribute to metal chelating ability of peptides (Saiga et al., 2003; Zhang et al., 2010). In addition, Decker, Crum, and Calvert (1992) reported that specific peptide structures and amino acid side chain residues greatly influence the ability to chelate metal ions. His is frequently observed in sequences of peptide ion chelation, especially when located at their N-termini (Chen, Muramoto, Yamauchi, Fujimoto, and Nokihara, 1998).

Among the reactive oxygen species (ROS), the hydroxyl radical is the most reactive as it damages biomolecules, such as amino acids, proteins, DNA and membrane components (Je, Lee, Lee, and Ahn, 2009). In addition, the superoxide radical is very harmful to cellular components, acting as a precursor for more ROS, including singlet oxygen and hydroxyl radicals (Aurand, Boonme, and Gidding, 1977). Fraction B1 did not show hydroxyl radical scavenging activity at peptide concentrations of 0.1-0.2 mg/ml (Figure 4.5a), but the activity was noticed at peptide concentration of 0.3 mg/ml and activity increased with peptide concentration up to 0.5

mg/ml (Figure 4.5a). Limited hydroxyl radical scavenging activity of fraction B1 could be attributed to its larger molecular mass as compared to the other 2 fractions. In addition, fraction B1 contained much more peptide sequences (282 peptides) than others, leading to a dilution effect of the peptides exhibiting hydroxyl radical scavenging activity. The hydroxyl radical scavenging activity of fractions B2 and B3 increased with peptide concentrations (Figures 4.5b-c). Although ABTS scavenging activity and FRAP of fraction B2 was lower than those of fraction B3, its metal chelating ability and hydroxyl radical scavenging ability were higher ($p < 0.05$, Table 4.2). This implies that peptides possessing ABTS scavenging activity and FRAP were different from those with metal chelating and hydroxyl radical scavenging ability in term of amino sequence and composition. The purified peptide isolated from the Alaska pollock (*Theragra chalcogramma*) frame protein hydrolysate, LPHSGY, scavenged 35% of the hydroxyl radicals at 53.6 μM (Je, Park, and Kim, 2005). Chen et al. (1998) suggested that His-containing peptides act as hydroxyl radical scavengers. Three of the dipeptides from royal jelly protein contain Tyr at their C-termini (KY, RY and YY) and have strong hydroxyl radical scavenging activity because the phenolic group of Tyr can scavenge free radicals by donating a hydrogen atom from its hydroxyl group (Guo et al., 2009). Rajapakse, Mendis, Jung, Je, and Kim (2005) suggested that higher radical scavenging activity was observed when peptides contained two Phe and two His residues.

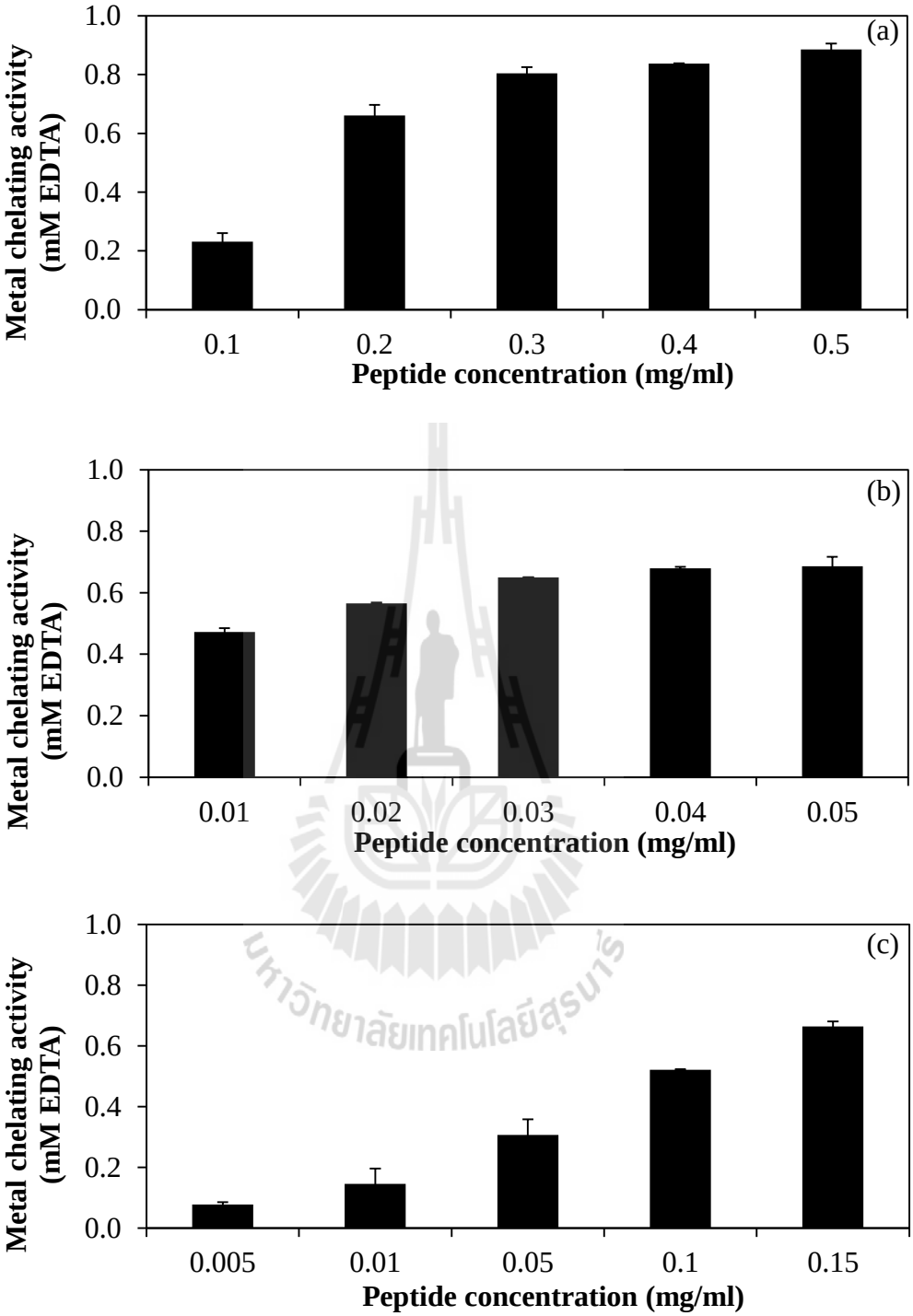

Figure 4.4 The effect of the peptide concentration on the metal chelating activity of partially-purified peptides, fractions B1 (a), B2 (b) and B3 (c) obtained by chromatography using the Superdex 30 prep grade gel. Values are expressed as the mean ± S.D. (n=2).

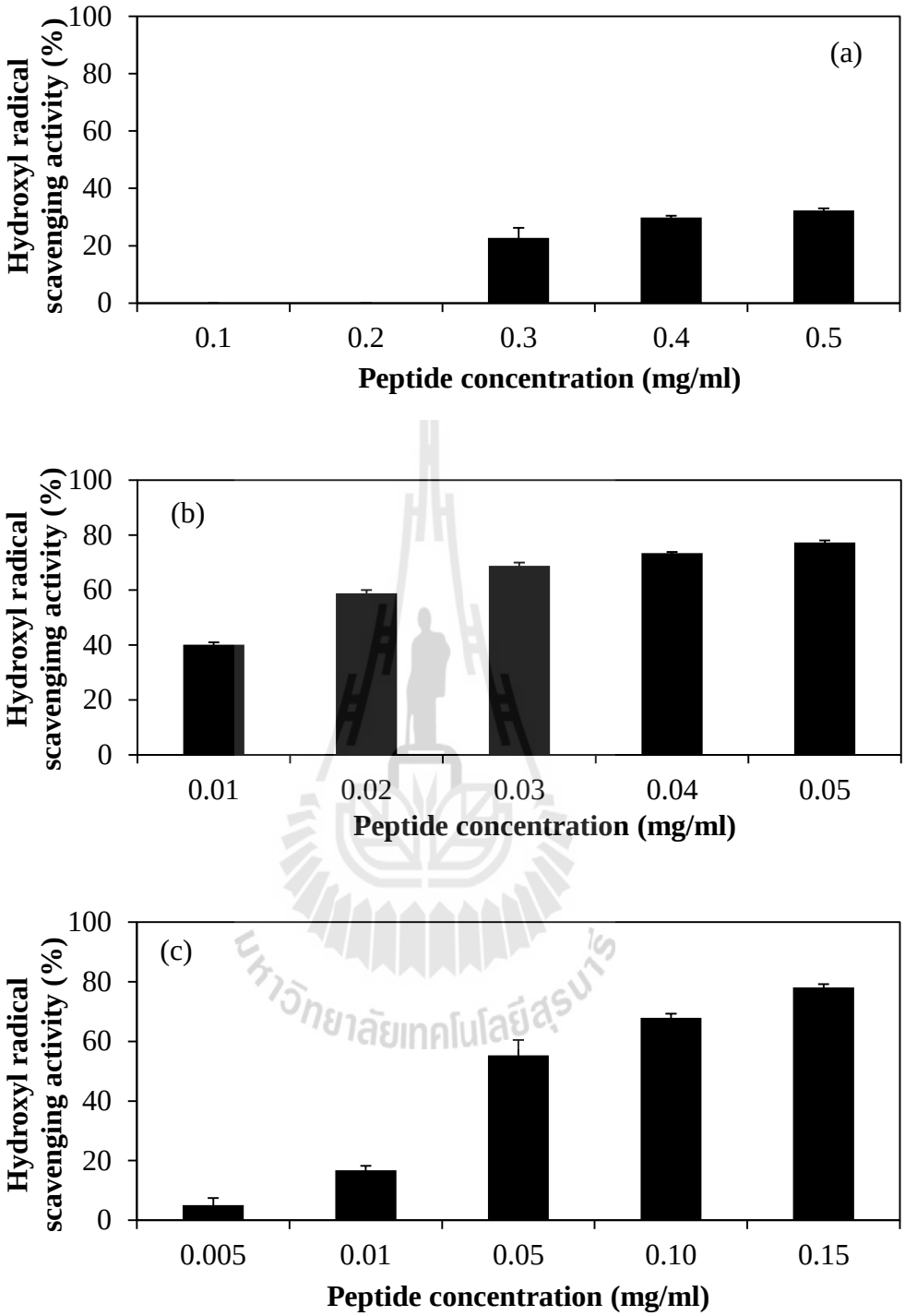

Figure 4.5 The effect of the peptide concentration on the hydroxyl radical scavenging activity of partially-purified peptide, fractions B1 (a), B2 (b) and B3 (c) obtained by chromatography using the Superdex 30 prep grade gel. Values are expressed as the mean ± S.D. (n=2).

Since fraction B3 exhibited high ABTS scavenging activity and FRAP as well as the highest peptide yield was obtained, peptides of fraction B3 from *de novo* sequencing were synthesized and evaluated for antioxidant activity. One of the synthetic peptides, NTFLFFK, was not soluble in water. A peptide containing Lys (K), Thr (T) and Asn (N) is likely to form intermolecular hydrogen bonds. In addition, three residues of Phe (F) promoted hydrophobic interactions, particularly in the lyophilized form. Only the FLGSFL**Y**E**Y**SR peptide showed an ABTS radical scavenging activity, and no synergistic effects were observed when mixed with other synthetic peptides (Table 4.4). Aliaga and Lissi (2000) reported that amino acids demonstrated the ABTS radical scavenging reactivity followed the order Cys >> Trp > Tyr > His, and a labile hydrogen atom (e.g. SH, NH, and OH) was required for the reaction to occur. Hernández-Ledesma et al. (2009) reported that Trp and Cys residues in lunasin peptide fragments were responsible for their ABTS radical scavenging activity. The presence of three Cys (C) residues in CFCTKPC was believed to contribute to its high ABTS radical scavenging activity (Huang et al. 2012). Localization of Cys residues at both the N- and C-terminal position allowed the accessibility to radicals in an easier manner. Peptides from α-lactalbumin containing either Tyr or Trp at one of the extremities of the sequence displayed the most efficient ABTS radical scavenging activity (Sadat et al., 2011). Gu et al. (2012) reported that Tyr residue at the N-terminus of **Y**ECG contributed to its ABTS radical scavenging ability. In addition, the carboxyl of Glu (E) located next to Tyr could induce the release of hydrogen atom of phenolic hydroxyl in Tyr, enhancing the antioxidant activity. In our study, the high ABTS radical scavenging activity of FLGSFL**Y**E**Y**SR peptide would be likely attributed to two Tyr residues in the

sequence. It can be speculated that the absence of ABTS radical scavenging activity of other synthetic peptides could be due to the lack of Tyr, Trp, His and Cys residues in their sequences. Therefore, FLGSFL**Y**E**Y**SR was considered to be a main antioxidant peptide of fraction B3.

Differences in ABTS scavenging activity between the mixture of synthetic peptides (P1+P2+P3+P4) and fraction B3 peptides could be due to from several reasons. The mixture of synthetic peptides with regard to individual concentration, which was 1.25 mg/ml, might not totally represent individual concentration contained in fraction B3. In addition, *de novo* peptide sequencing might not capture all important antioxidant peptides.

Attempts were also made to determine other antioxidant activities of individual and mixed synthetic peptides. The FLGSFLYEYSR showed FRAP value of 22.045±1.323 µM at a very high concentration of 25 mg/ml, indicating its low reducing power. In addition, FRAP values of other synthetic peptides were negligible even at 25 mg/ml. For this reason, FRAP assay of mixed synthetic peptides was not determined. In addition, all synthetic peptides (P1-P5) showed relatively low metal chelating activity and hydroxyl radical scavenging activity at 5 mg/ml. The possibility of synergistic effects of multiple peptides that could reduce Fe^{3+}-TPTZ, scavenge hydroxyl radical, and chelate metal ion might have contributed to higher antioxidant activity in Fraction B3 than those synthetic peptides. In addition, it is possible that, a number of characteristics including, functional groups, the amphiphilic nature and solubility of the mixture of peptides present in fraction B3 could have been distinctly different from the characteristics of the single peptides that were chemically synthesized.

Table 4.4 The ABTS radical scavenging activity (mM Trolox) of individual and mixed synthetic peptides obtained from *de novo* sequencing of fraction B3 at a total concentration of 5 mg/ml.

Peptide	ABTS radical scavenging activity (mM Trolox)
P1	0^{a}
P1+P2	0^{a}
P1+P3	0.015 ± 0.015^{a}
P1+P2+P3	0.005 ± 0.000^{a}
P1+P2+P4	2.140 ± 0.024^{c}
P1+P2+P3+P4	1.962 ± 0.021^{b}
P2	0^{a}
P2+P3	0^{a}
P2+P4	2.287 ± 0.029^{d}
P2+P3+P4	2.146 ± 0.005^{c}
P3	0^{a}
P3+P4	2.281 ± 0.011^{d}
P1+P4	2.290 ± 0.003^{d}
P1+P3+P4	2.125 ± 0.019^{c}
P4	2.448 ± 0.003^{e}
P5	0^{a}

Note: *Concentration of individual peptides was set equally in each mixture to attain total concentration of 5 mg/ml, i.e. the mixture of 2, 3, and 4 peptides composed of each peptide at 2.5, 1.67, and 1.25 mg/ml, respectively.

**P1 is VELLVPK, P2 is AGNQVLNLQADLPK, P3 is LGTGTDL, P4 is FLGSFLYEYSR and P5 is NTFLFFK.

*** P5 was only soluble in trifluoroacetic acid and it precipritated when mixed with other synthetic peptides. Therefore, synergistic effects of this peptide can not be performed.

****Different letters indicate significant differences among the means ($P < 0.05$). Values are expressed as the mean ± S.D. (n = 2).

4.4.3 Cytoprotective effect

Since fractions B2 and B3 contained high salt which interfered with cytoprotective studies, only fraction B1 and the synthetic FLGSFLYEYSR peptide representing an antioxidant peptide from fraction B3 were determined for their cytoprotective effect. DCFH-DA is widely used to measure oxidative stress in cells due to the high sensitivity of this fluorescence-based assay (Bonini, Rota, Tomasi, and Mason, 2006). DCFH-DA penetrates into the cells and is hydrolyzed to DCFH by intracellular esterases. The presence of ROS can oxidize DCFH to form DCF, a fluorescent product. The fluorescent intensity of the cells treated with 0.3 mM H_2O_2 increased approximately 1.4-fold compared to the control (without H_2O_2) (Figure 4.6). However, the pretreatment with fraction B1 and the synthetic FLGSFLYEYSR peptide from fraction B3 decreased the DCF fluorescent intensity. Peptides from fraction B1 and the synthetic FLGSFLYEYSR peptide obtained from fraction B3 showed comparable DCF fluorescent intensity ($p>0.05$) at concentrations up to 20 µg/ml. This result suggested that fraction B1 and a synthetic peptide selected from the pooled *de novo* peptides of fraction B3, FLGSFLYEYSR, had a cellular radical scavenging effect.

It is well known that H_2O_2 itself is not highly reactive; however, it forms highly reactive oxygen species ($^{\bullet}OH$) in the presence of transition metal ions and through other mechanisms (Halliwell and Gutteride, 1992). The formation of $^{\bullet}OH$ and other ROS initiates lipid peroxidation and causes DNA damage (Zhang, Wang, Xu, and Gao, 2009). Je, Qian, Lee, Byun, and Kim (2008) reported that the purified peptide isolated from the bigeye tuna, LNLPTAVYMVT, had a considerable radical scavenging effect in HT1080 cells at a concentration of 50 µg/ml. The potent radical

scavenging effects were due to the presence of Pro, Leu, Ala, and Tyr. Zhang et al. (2010) reported that a purified peptide isolated from rice endosperm, FRDEHKK, can scavenge intracellular ROS in mouse macrophages, RAW 264.7 cells. Liu, Wang, Duan, Guo, and Tang (2010) reported that three purified peptides from water buffalo horn, QYDQGV, YED**C**TDCGN and AADNANELFPPN, protected rat cerebral microvascular endothelial cells (rCMECs) against H_2O_2-induced injury. The authors suggested that Tyr, Gly, Cys and Phe-Pro-Pro (FPP) residues may play an important role in scavenging radicals through direct electron transfer. They also suggested that hydrophobic amino acids, such as Gly, Ala, Val, Leu, Trp, Pro and Phe, might increase an affinity and reactivity towards the cell membrane in the rCMECs, leading to a better antioxidant efficacy.

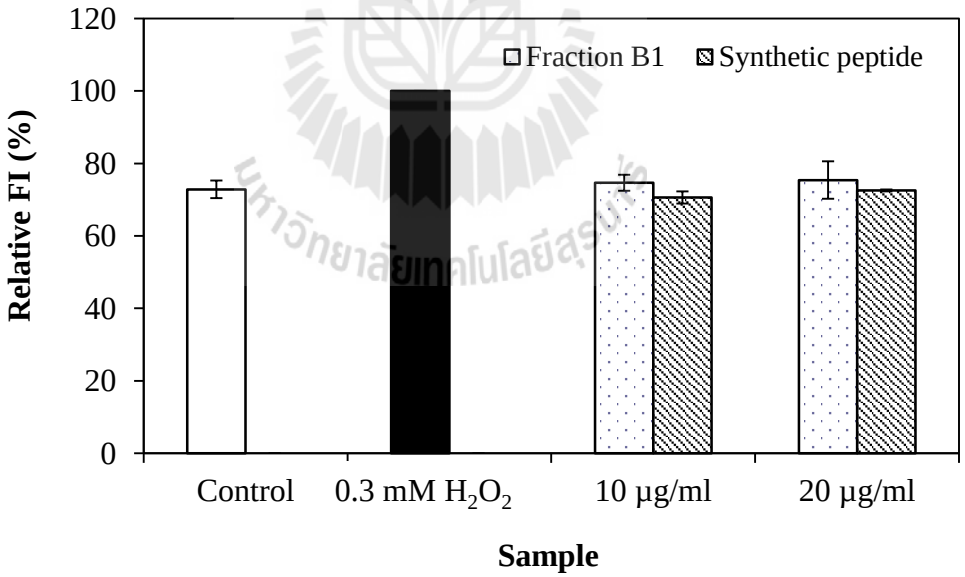

Figure 4.6 The cellular radical scavenging activities of fraction B1, which was obtained by chromatography using the Superdex 30 prep grade gel, and of the

(Figure 4.6 continued)

synthetic FLGSFLYEYSR peptide. HepG2 cells were labeled with 10 µM fluorescence dye (DCFH-DA) and treated with either peptide fraction B1 (10 and 20 µg equivalent/ml) or with the synthetic FLGSFLYEYSR peptide (20 µg/ml). Fluorescence intensities of DCF due to oxidation of DCFH by cellular ROS (generated by H_2O_2) were detected at E_x=485 nm and E_m=530 nm. The results were calculated as the percentage of relative fluorescence intensity (FI) of H_2O_2.Values are expressed as the mean ± S.D. (n=4).

4.5 Conclusions

Fraction B3 obtained from SEC of FBS hydrolysate showed the highest ABTS radical scavenging activity and FRAP value, while fraction B2 exhibited the highest metal chelating activity and hydroxyl radical scavenging activity. Antioxidant activities of all fractions were concentration-dependent. The FLGSFLYEYSR appeared to be one of important antioxidant peptides in fraction B3, which scavenged both synthetic radicals and cellular ROS. Although fraction B1 showed moderate antioxidant activities towards synthetic radicals, it protected HepG2 cell against H_2O_2-induced oxidative damage at concentration as low as 10 µg/ml.

4.6 References

Aliaga, C. and Lissi, E. (2000). Reactions of the radical cation derived from 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) with amino acids. Kinetics and mechanism. **Canadian Journal of Chemistry.** 78: 1052-1059.

Ames, B. M., Shigena, M. K., and Hagen, T. M. (1993). Oxidants, antioxidants and

the degenerative diseases of aging. **Proceedings of the National Academy of Science**. 90: 7915-7922.

Aurand, L. W., Boone, N. H., and Giddings, G. G. (1977). Superoxide and singletoxigen in milk peroxidation. **Journal of Dairy Science.** 60: 363-369.

Bonini, M. G., Rota, C., Tomasi, A., and Mason, R. P. (2006). The oxidation of 2',7'-dichlorofluorescin to reactive oxygen species: A self-fulfilling prophesy? **Free Radical Biology and Medicine.** 40: 968-975.

Chen, H. M., Muramoto, K., Yamauchi, F., Fujimoto, K., and 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.

Decker, E. A., Crum, A. D., and Calvert, J. T. (1992). Differences in the antioxidant mechanism of carnosine in the presence of copper and iron. **Journal of Agricultural and Food Chemistry.** 40: 756-759.

Decker, E. A. and Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. **Journal of Agricultural and Food Chemistry**. 38: 674-677.

Gu, L., Zhao, M., Li, W., You, L., Wang, J., and Wang, H. (2012). Chemical and cellular antioxidant activity of two novel peptides designed based on glutathione structure. **Food and Chemical Toxicology.** 50: 4085-4091.

Guo, H., Kouzuma, Y., and Yonekura, M. (2009). Structures and properties of antioxidative peptides derived from royal jelly protein. **Food Chemistry**. 113: 238-245.

Halliwell, B. and Gutteridge, J. M. C. (1992). Biologically relevant metal ion-dependent hydroxyl radical generation.Anupdate. **FEBS Letters.** 307: 108-112.

Halliwell, B., Aeschbach, R., Loliger, J., and Aruoma, O.I. (1995). The characterization of antioxidants. **Food and Chemical Toxicology.** 33: 601-617.

He, R., Girgih, A. T., Malomo, S. A., Ju, X., and Aluko, R. E. (2013). Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. **Journal of Functional Foods**. 5: 219-227.

Hernández-Ledesma, B., Davalos, A., Bartolome, B., and Amigo, L. (2005). Preparation of antioxidant enzymatichydrolysates from α-lactalbumin and β-lactoglobulin.Identification of active peptides by HPLC-MS/MS. **Journal of Agricultural and Food Chemistry**. 53: 588-593.

Hernández-Ledesma, B., Hsieh, C. C., and de Lumen, B. O. (2009). Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7macrophages. **Biochemical and Biophysical Research Communications.** 390: 803-808.

Huang, G. J., Deng, J. S., Chen, H. J., Huang, S. S., Liao, J. C., Hou, W. C., and Lin, Y. H. (2012). Defensin protein from sweet potato (*Ipomoea batatas* [L.] Lam 'Tainong 57') storage roots exhibits antioxidant activities *in vitro* and *ex vivo*. **Food Chemistry.** 135: 861-867.

Je, J. Y., Lee, K. H., Lee, M. H., and Ahn, C. B. (2009). Antioxidant and antihypertensive protein hydrolysates produced from tuna liver by enzymatic hydrolysis. **Food Research International.** 42: 1266-1272.

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

Je, J. Y., Qian, Z. J., Byun, H. G., and Kim, S. K. (2007). Purification and characterization

of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis. **Process Biochemistry.** 42: 840-846.

Je, J. Y., Qian, Z. J., Lee, S. H., Byun, H. G., and Kim, S. K. (2008). Purification and antioxidant properties of bigeye tuna (*Thunnus obesus*) dark muscle peptide on free radical-mediated oxidative systems. **Journal of Medicinal Food.** 11: 629-637.

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

Kudo, K., Onodera, S., Takeda, Y., Benkeblia, N., and Shiomi, N. (2009). Antioxidative activities of some peptides isolated from hydrolyzed potato protein extract. **Journal of Functional Foods**. 1: 170-176.

Leanderson, P., Faresjo, A. O., and Tagesson, C. (1997). Green tea polyphenols inhibit oxidant-induced DNA strand breakage in cultured lung cells. **Free Radical Biology and Medicine**. 23: 235-242.

Li, B., Chen, F., Wang, X., Ji, B., and Wu, Y. (2007). Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization–mass spectrometry. **Food Chemistry.** 102: 1135-1143.

Liu, R., Wang, M., Duan, J. A., Guo, J. M., and Tang, Y. P. (2010). Purification and identification of three novel antioxidant peptides from *Cornu bubali* (water buffalo horn). **Peptides.** 31: 786-793.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. **Journal of Biological Chemistry.** 193: 265-275.

Ngo, D. H., Qian, Z. J., Ryu, B., Park, J. W., and Kim, S. K. (2010). *In vitro* antioxidant activity of a peptide isolated from Nile tilapia (*Oreochromis niloticus*) scale gelatin in free radical-mediated oxidative systems. **Journal of Functional Foods.** 2: 107-117.

Qian, Z. J., Jung, W. K., Byun, H. G., and Kim, S. K. (2008). Protective effect of an antioxidativepeptide purified from gastrointestinal digests of oyster, *Crassostrea gigas* against free radical induced DNA damage. **Bioresource Technology**. 99: 3365-3371.

Raghavan, S., Kristinsson, H. G., and Leeuwenburgh, C. (2008). Radical scavenging and reducing ability of tilapia (*Oreochromis niloticus*) protein hydrolysates. **Journal of Agricultural and Food Chemistry.** 56: 10359-10367.

Rajapakse, N., Mendis, E., Jung, W. K., Je, J. Y., and Kim, S. K. (2005). Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. **Food Research International.** 38: 175-182.

Saiga, A., Tanabe, S., and Nishimura, T. (2003). Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. **Journal of Agricultural and Food Chemistry.** 51: 3661-3667.

Sakanaka S., and Tachibana, Y. (2006). Active oxygen scavenging activity of egg-yolk protein hydrolysates and their effects on lipid oxidation in beef and tuna homogenates. **Food Chemistry**. 95: 243-249.

Sadat, L., Cakir-Kiefer, C., N'Negue, M. A., Gaillard, J. L., Girardet, J. M., and Miclo, L. (2011). Isolation and identification of antioxidative peptides from bovine α-lactalbumin. **International Dairy Journal.** 21: 214-221.

Shiraishi, H., Kataoka, M., Morita, Y., and Umemoto, J. (1993). Interactions of hydroxyl

radicals with Tris (hydroxymethyl) aminomethane and good's buffers containing hydroxymethyl or hydroxyethyl residues produce formaldehyde. **Free Radical Research Communications**. 19: 315-321.

Sinsuwan, S., Rodtong, S., and Yongsawatdigul, J. (2008). Production and characterization of NaCl-activated proteinases from *Virgibacillus* sp. SK33 isolated from fish sauce fermentation. **Process Biochemistry**. 43: 185-192.

Stohs, S. J. and Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal ions. **Free Radical Biology and Medicine.** 18: 321-336.

Sun, L., Zhang, Y., and Zhuang, Y. (2013). Antiphotoaging effect and purification of an antioxidant peptide from tilapia (*Oreochromis niloticus*) gelatin peptides. **Journal of Functional Foods.** 5: 154-162.

Vitaglione, P., Morisco, F., Caporaso, N., and Fogliano, V. (2004). Dietary antioxidant compounds and liver health. **Critical Reviews in Food Science and Nutrition.** 44: 575-586.

Wiriyaphan, C., Chitsomboon, B., and Yongsawadigul, J. (2012). Antioxidant activity of protein hydrolysates derived from threadfin bream surimi byproducts. **Food Chemistry.** 132: 104-111.

Zhang, S., Wang, Z., Xu, S., and Gao, X. (2009). Purification and characterization of a radical scavenging peptide from rapeseed protein hydrolysates. **Journal of the American Oil Chemists' Society.** 86: 959-966.

Zhang, J. H., Zhang, H., Wang, L., Guo, X. N., Wang, X. G., and Yao, H. Y. (2010). Isolation and identification of antioxidative peptides from rice endosperm protein enzymatic hydrolysate by consecutive chromatography and MALDI-TOF/TOF MS/MS. **Food Chemistry.** 119: 226-234.

CHAPTER V

ANTIOXIDANT PROPERTIES OF ULTRAFILTRATION FRACTIONS OF THREADFIN BREAM SURIMI BYPRODUCT HYDROLYSATES

5.1 Abstract

Protein hydrolysate from frame, bone and skin (FBSH) of threadfin bream was prepared using *Virgibacillus* sp. SK33 proteinase and fractionated using sequential ultrafiltration membranes with molecular weight cut-off (MWCO) of 30, 5 and 1 kDa, respectively. Four fractions, namely FBSH-I (>30 kDa), FBSH-II (5-30 kDa), FBSH-III (1-5 kDa), and FBSH-IV (<1 kDa), were obtained. Amino acid composition, surface hydrophobicity, and antioxidant activities based on different chemical and biological assays of all fractions were studied. All fractions were rich in Lys, Glu/Gln, Gly, Pro, Ala, Asp/Asn, and Arg. FBSH-III and FBSH-IV showed the highest surface hydrophobicity measured by the fluorescence probe 8-anilino-1-naphthalenesulfonic acid (ANS) ($p<0.05$). FBSH-III showed the highest antioxidant activity and cytoprotective effects against *tert*-butylhydroperoxide (TBHP)-induced cytotoxicity of Caco-2. In addition, FBSH-III inhibited lactate dehydrogenase (LDH) leakage and intracellular reactive species (ROS) production, depending on peptide concentration. FBSH-III retained antioxidant activity after various thermal (40-121°C) and pH treatments (pH 2.0-10.0) as well as *in vitro* simulated gastrointestinal digestion. In

addition, cytoprotective effect of FBSH III did not change after Gastrointestinal (GI) digestion. These results suggested that FBSH-III might be potential neutraceutical peptides with antioxidative properties.

Keywords: Threadfin bream, protein hydrolysate, antioxidant activites, ultrafiltration, cytoprotective effect.

5.2 Introduction

In aerobic organisms, reactive oxygen species (ROS) are produced during normal physiological processes, such as energy production, metabolism or generation of defenses against invasive microorganisms (Cornelli, 2009). The production of various ROS is closely related with the participation of redox-active metals (e.g. Fe^{2+}, Cu^{+} and others) (Valko, Morris, and Cronin 2005). The harmful effects of ROS are balanced by the antioxidant action, both antioxidant enzymes and non-enzymatic antioxidants (Halliwell, 1996; Valko et al., 2007). Oxidative stress occurs when ROS overload the body's antioxidant defenses or when the antioxidant defense system loses its capacity for response. Excessive ROS can damage DNA, proteins, and lipids, leading to many serious diseases like cancers and inflammatory (Calabrese et al., 2005; Simmonds, and Rampton, 1993). Therefore, diet-derived natural antioxidants that enhance or balance antioxidative defenses in the body have been continually sought.

It has been reported that protein hydrolysates from threadfin bream surimi byproducts prepared using *Virgibacillus* sp. SK33 proteinases showed antioxidant activities against synthetic free radicals and cytoprotective effect induced by *tert*-butyl hydroperoxide (TBHP) in HepG2 cell line (Wiriyaphan, Chitsomboon, and

Yongsawadigul, 2012). In addition, in our previous study, fractionation of antioxidant peptides from an FBS hydrolysate prepared from *Virgibacillus* sp. SK33 proteinase using ion exchange and size exclusion chromatography could improve antioxidant activity. However, chromatographic technique is relatively high cost and low yield, limiting the application in the industry. Ultrafiltration membranes technique is simple and economic processes that have been successfully used to concentrate antioxidant peptides from soy protein (Moure, Domínguez, and Parajó, 2006). In addition, ultrafiltration technique has the major advantage that the molecular weight distribution of the desired hydrolysates can be controlled by adoption of an appropriate UF membrane (Je, Qian, Byun, and Kim, 2007). Application of non-purified protein hydrolysate can have certain benefits over those of purified peptides since the absorption of oligopeptides can be increased in the presence of amino acids and other constituents presenting in the hydrolysate (Pappenheimer and Volpp, 1992). Investigation the antioxidant efficacy of fractionated peptides would, therefore, be more relevant than the purified peptides as far as food application is concerned.

Stability of antioxidant peptides during food processing should be assessed. Nalinanon, Benjakul, Kishimura, and Shahidi (2011) reported that a small loss in antioxidant activity of peptides might be due to either degradation or aggregation, caused by heat treatment. In addition, antioxidant peptides might undergo conformation changes at high alkaline and acidic pHs, leading to the loss in their antioxidant ability (Klompong, Benjakul, Kantachote, Hayes, and Shahidi, 2008). Furthermore, when peptides pass through the gastrointestinal (GI) tract, they could be modified through GI proteases, which could consequently alter antioxidant properties (Chen and Li, 2012). Although animal studies and human clinical trials are the best

ways to study the bioactivity and bioavailability of functional ingredients, *in vitro* simulated GI digestion is being widely used. It allows for rapid and inexpensive study of bioactive compounds for assessment of their efficacy in *vivo* (Cinq-Mars, Hu, Kitts, and Li-Chan, 2008). Therefore, effects of thermal, pH, and *in vitro* GI tract model on oxidative stability of antioxidant peptides should be monitored.

Due to the wide variety of oxidation processes and antioxidant action of protein hydrolysates or peptides, the use of a single method to evaluate the antioxidant activity cannot provide a clear picture about its antioxidant potential (Ktari et al., 2012). Therefore, antioxidant activity could be determined using various assays. Peptides with pronounced antioxidant activity *in vitro* based on synthetic radical scavenging assays may exert little or no activity *in vivo*. A step closer to *in vivo* is the use of the cellular model system.

Caco-2 cells derived from a human intestinal adenocarcinoma have recently been found to provide a useful cell culture model of the small intestinal epithelium, including investigations on responses to 'oxidative stress' and nutritional antioxidants (Ekelund et al., 2005). Under standard culture conditions, the Caco-2 cell line exhibits spontaneous enterocyte-like differentiation, showing morphological polarity and expressing brush-border hydrolases (Hidalgo, Raub, and Borchardt, 1989). Therefore, determination of antioxidant activity in Caco-2 cells represents a more biologically relevant method than chemical assays of antioxidant activity, because it accounts for some aspects of the uptake, metabolism, and location of antioxidants within cells (Carrasco-Castilla et. al, 2012). *Tert*-butyl hydroperoxide (TBHP), has been widely used to induce oxidative cell injury (Aherne and O'Brien, 2000; Alía, Ramos, Mateos, Bravo, and Goya, 2005). Decomposition of TBHP, aiding by metal ions and their

complexes, will accelerate lipid peroxidation chain reactions and induce cell toxicity by damaging DNA.

Objectives of this study were to determine antioxidant activity of ultrafiltration fractions of FBS hydrolysate, using cellular (Caco-2 cells) and synthetic chemical systems. As biomarkers for antioxidant capacity, the intracellular ROS level- (DCFH-DA assay), and cytotoxicity-induced by TBHP were used. In addition, effect of thermal and pH treatments, as well as *in vitro* GI digestion on oxidative stability of antioxidant peptides were assessed.

5.3 Materials and methods

5.3.1 Materials

FBS from a deboning process of threadfin bream surimi production was provided by Andaman Surimi Industry (Samutsakorn, Thailand). The sample was kept in ice and transported to Suranaree University of Technology laboratory. Up on arrival, sample was dried, ground, and defatted as described in Chapter III (section 3.3.1) and kept at -20°C until use. *Virgibacillus* sp. SK 33 extracellular proteinase was prepared as described in Chapter III (section 3.3.2). 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were purchased from BioChemika (Buchs, Switzerland). Trinitrobenzenesulfonic acid (TNBS), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), 2-deoxy D-ribose, amino acid standard, L-Cysteic acid, methionine sulfone, Tryptophane (98% by TLC) and the internal standard l-norleucine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Caco-2 cell human hepatoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA).

Dulbecco's Modified Eagle Media (DMEM) was purchased from Gibco BRL, Life Technologies (Gaithersburg, MD, USA). Other chemicals and reagents were of analytical grade.

5.3.2 Preparation and fractionation of FBS hydrolysate

Defatted FBS powder (90 g) was added 900 mL deionized water and pH was adjusted to pH 8.0 using 1 N NaOH. *Virgibacillus* sp. SK33 proteinase was added at 13.5 U/g FBS powder and hydrolytic reaction was carried out at 65°C for 16 h. Subsequently, the hydrolysates were heated at 90°C for 10 min to inactivate proteinase and centrifuged at 10,000 g for 20 min at 4°C. Supernatants were referred to as FBS hydrolysates (FBSH). FBSH was further fractionated using ultrafiltration membranes with MWCO of 30, 5 and 1 kDa, respectively, in the sequential order. Fractions were designated as follows: FBSH-I, the retentate of the 30-kDa membrane (>30 kDa); FBSH-II, retentate of the 5-kDa membrane obtained from the 30-kDa permeate (5-30 kDa); FBSH-III, retentate of the 1-kDa membrane obtained from the 5-kDa permeate (1-5 kDa); and FBSH-IV, the permeate of the 1-kDa membrane (<1 kDa). FBSH and its ultrafiltrated fractions were lyophilized. The peptide contents were determined using the TNBS method using leucine as a standard as described in Chapter III (section 3.3.5.1). Peptide yield of each fraction was calculated based on α-amino acid content of FBSH. Amino acid composition, surface hydrophobicity, and antioxidant activities in cellular and synthetic radicals of each fraction were determined.

5.3.3 Chemical characteristics of ultrafiltrated fractions

5.3.3.1 Amino acid composition

Samples were hydrolyzed following AOAC (2000). In brief, lyophilized

powder of FBSH and ultrafiltrated fractions (0.05-0.10 g) was digested with 6 N HCl at 110°C for 24 h under nitrogen. The cysteine and methionine contents were determined using performic acid oxidation with acid hydrolysis-sodium metabisulfite method, while the tryptophan content was determined after alkaline hydrolysis. Norleucine was used as internal standard. Quantitative analysis of total amino acids was achieved by a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge Science Park, England). The method involves a post-column derivatization of the amino acids with ninhydrin, which can be determined spectrophotometerically at 570 and 440 nm. The amino acid was expressed as g/100 g protein.

5.3.3.2 Surface hydrophobicity (Ho)

Surface hydrophobicity (Ho) was measured using the fluorescence probe 8-anilino-1-naphthalene sulfonate (ANS) according to the method of Tang, Peng, Zhen, and Chen (2009) with some modification. Stock solutions of protein hydrolysates (0.05% w/v) were prepared in 0.01 M phosphate buffer, pH 7.0. Serial dilutions in 0.01 M phosphate buffer of to obtain protein content ranging from 0.001 to 0.02% (w/v) were prepared. Protein content was calculated based on total amino acid content. Ten µl of 8.0 mM of ANS solution was added to 2 ml of each dilution and incubated in the dark for 10 min. Subsequently, fluorescence intensity (FI) of the mixture was monitored at 374 nm (excitation) and 485 nm (emission) using JASCO FP-8000 Series fluorescence spectrofluorometers (Model 8300FP, Hachioji, Tokyo, Japan). The initial slope of the FI versus concentration (% w/v) plot was used as an index of Ho.

5.3.3.3 Size exclusion chromatography (SEC)

Molecular weight (MW) distribution of sample was determined using size

exclusion chromatography. The lyophilized of FBSH and ultrafiltrated fractions was dissolved in deionized water (1.6 mg leucine equivalents/ml). A 10 µl of reconstituted peptide was loaded into a Superdex Peptide 10/300 GL column (10 mm x 300 mm, exclusion limit: 2×10^4, separation range: 100-7000 Da, void volume which calibrated with blue dextran: 6.3 ml); GE Healthcare, Piscataway, NJ, USA) pre-equilibrated with deionized water. Elution was carried out at a flow rate of 0.3 ml/min using 1 column volume of deionized water. Synthetic peptide, AGNQVLNLQADLPK (1481 Da), hippuryl L-histidyl-L-leucine (430 Da) and tyrosine (182 Da) were used as standards. Absorbance was monitored at 215 nm.

5.3.4 Antioxidant activity

5.3.4.1 ABTS radical scavenging activity assay

Lyophilized samples were dissolved in deionized water at various peptide concentrations (0.5, 1.0, 1.5, and 2.0 mg/ml). The ABTS assay was determined as described in Chapter III (section 3.3.5.2.1). Results were expressed as mM Trolox equivalents.

5.3.4.2 Ferric reducing antioxidant power assay (FRAP assay)

Lyophilized samples were dissolved in deionized water at various peptide concentrations (1, 2, 3, and 5 mg/ml). The FRAP assay was determined as described in Chapter III (section 3.3.5.2.2). Results were expressed as µM Trolox equivalents.

5.3.4.3 Metal chelating assay

Lyophilized samples were dissolved in deionized water at various peptide concentrations (1, 2, 3, and 5 mg/ml). Metal chelating assay was determined as described in Chapter IV (section 4.3.6.3). Results were expressed as mM Ethylene-

diaminetetraacetic acid (EDTA) equivalents.

5.3.4.4 Hydroxyl radical scavenging activity assay

Lyophilized samples were dissolved in deionized water at various concentrations (1, 2, 3, and 5 mg/ml). Hydroxyl radical scavenging activity assay was determined as described in Chapter IV (section 4.3.6.4). Results were expressed as percentage of inhibition compared to the control. A control was prepared using 100 µl of deionized water instead of the peptide solution.

5.3.5 Cytotoxicity/cytoprotective effect

5.3.5.1 Cell culture

Caco-2 cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 1% non-essential amino acid and, 1% penicillin-streptomycin, and incubated at 37 °C in a humidified atmosphere with 5% CO_2. When cells were reached 90% confluence, they were subcultured by trypsination.

5.3.5.2 TBHP-induced cytotoxicity on Caco-2 cells

Cells were seeded in a 96-well plate (1.5×10^4 cells per well) and incubated in a humidified incubator containing 5% CO_2 and 95% air at 37°C for 24 h. Then, cells were treated with various concentrations of TBHP (at final concentration of 0.2-1.0 mM) and incubated for 4 h. The culture medium was removed and replaced with a fresh medium containing 100 µl of 0.5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and further incubation for 2 h. The culture medium was discarded and the purple formazan crystals were dissolved with dimethyl sulfoxide (DMSO) (100 µl/well). Absorbance was monitored at 570 nm using a microplate reader (Biotek Instrument Inc., Vermont, USA). The result was expressed as a percentage of viable cells compared to the control culture (without TBHP). The

final concentration at 0.5 mM was selected for further experiment described in section 5.3.5.3.

5.3.5.3 Cytoprotective effect of ultrafiltrated fractions on TBHP-induced cytotoxicity

Cells were seeded in a 96-well plate (1.5×10^4 cells per well) and incubated in a humidified incubator containing 5% CO_2 and 95% air at 37 °C for 24 h. Then, cells were pretreated with the medium containing either FBSH or ultrafiltrated fractions at a final concentration of 200 µg/ml and incubated for 12 h. Subsequently, 10 µl of 10.5 mM TBHP (final concentration at 0.5 mM) was added and further incubated for 4 h. Cell viability was determined using MTT dye solution as described in section 5.3.5.2. The result was expressed as a percentage of viable cells compared to the control culture (without sample and TBHP).

5.3.5.4 Protective effect of FBSH-III on TBHP-induced cytotoxicity

FBSH-III showing the highest antioxidant activities based on chemical and biological assays was selected to investigate does-dependent response on TBHP-induced cytotoxicity of Caco-2 cell at various peptide concentration (25, 50, 100, 200 µg/ml) as described in section 5.3.5.3.

LDH leakage was also determined. Caco-2 cells were seed in a 6-well plate at a concentration of 4×10^5 cells/well. Cells were incubated in a humidified incubator containing 5% CO_2 and 95% air at 37°C for 24 h. Culture medium was removed and replaced with the medium containing different concentration of FBHS-III (25, 50, 100 and 200 µg/ml). After 12 h incubation with the peptide, the culture medium was removed. A 2 ml of 1 mM TBHP in DMEM was added, except for the control plate, and further incubated for 3 h. Subsequently, the culture medium was collected for

LDH assay. Cells were washed with 1 ml of PBS and were scraped in 0.2 ml of PBS and lysed on ice by sonication and centrifuged at 14,000 rpm for 25 min. Supernatant was collected for the analysis of LDH activity. LDH leakage was estimated by the method of Lima, Andrade, Seabra, Fernandes-Ferreira, and Pereira-Wilson (2005) with slight modifications. In brief, 700 µl of 5 mM pyruvate containing 0.095 mg/ml of NADH in 84 mM Tris-HCl pH 7.4 was mixed with either 0.3 ml of culture medium or diluted cell lysated and immediately read at 340 nm at 25 °C for 5 min. LDH activity in the culture medium was estimated using the following equation:

$$\text{LDH activity} = \frac{\text{LDH activity in the culture medium} \times 100}{(\text{LDH activity in the culture medium} + \text{LDH in lysated cell})}$$

5.3.5.5 Cellular ROS determination by 2', 7'-dichlorofluorescin diacetate (DCFH-DA) assay

A 200 µl of cells were seeded in fluorescence microtiter 96-well plates (1.5×10^4 cells per well) and incubated in a humidified incubator containing 5% CO_2 and 95% air at 37 ˚C for 24 h. Then, the culture medium was removed and cells were pretreated with a 200 µl of medium containing different concentration of FBSH-III (25, 50, 100 and 200 µg/ml) and incubated for 12 h. Culture medium was removed and washed with 100 µl of PBS (pH 7.4). Subsequently, 100 µl of 10 µM DCFH-DA in PBS (pH 7.4) was added and incubated in the dark for 30 min. Cells were washed with 100 µl of PBS (pH 7.4). Then, a 100 µl of 0.3 mM TBHP in PBS (pH 7.4) was added and incubated for 1 h. DCFH was oxidized by reactive oxygen species (ROS) to form 2', 7'-dichlorofluorescin (DCF), a fluorescent product, which was monitored at the excitation wavelength (E_x) of 485 nm and the emission wavelength (E_m) of 528

nm using a fluorescence microplate reader (Biotek Instrument Inc., Vermont, USA).

5.3.6 Stability of FBSH-III

5.3.6.1 Temperature stability

For temperature stability, lyophilized FBSH-III was dissolved in deionized water at peptide concentration of 1 mg/ml and incubated at various temperatures, 40, 60, 70, 80, 100 and 121 °C, for 1 h. Then, antioxidant activity of FBSH-III was assayed using ABTS radical scavenging activity. The result was expressed as relative activity (%) as compared to the untreated sample. The ABTS radical scavenging activity assay was chosen, because it was shown to be the most sensitive method studied (Brand-Williams, Cuvelier, and Berset, 1995).

5.3.6.2 pH stability

For pH stability, lyophilized sample was dissolved in deionized water at peptide concentration of 1 mg/ml. pH of sample was adjusted using either 6 N HCl or 6 N NaOH to 2.0, 4.0, 6.0, 8.0 or 10.0 and left at room temperature for 1 h. Subsequently, samples were adjusted pH to 7.4 using either 6 N HCl or 6 N NaOH Antioxidant activity of all samples was assayed using ABTS radical scavenging activity assay. The result was expressed as relative activity (%) as compared to the untreated sample (pH 7.4).

5.3.6.3 *In vitro* pepsin–pancreatin simulated GI digestion

Simulated GI digestion using an *in vitro* pepsin–pancreatin hydrolysis was performed as the method described by Sheih, Fang, and Wu (2009) with some modifications. The lyophilized FBSH-III was dissolved in deionized water (10% w/v) and adjusted pH to 2.0 using 6 N HCl. Then, pepsin was added with enzyme to substrate ratio of 1: 100 (w/w) and incubated at 37 °C for 3 h. The mixture was then

adjusted pH to 7.8 with 6 N NaOH. Neutralized suspension (1 ml) was centrifuged at 10,000 g for 10 min and the supernatant was used for determination of α-amino content using the TNBS method (Adler-Nissen, 1979) and antioxidant activity using ABTS radical scavenging activity. The remaining suspension was further digested with 2% (w/w) porcine pancreatin. After incubation in a 37 °C water bath for 4 h, the mixture was boiled at 90 °C for 10 min to terminate pancreatin activity and centrifuged at 10,000 g for 10 min. α-Amino content of the supernatant was determined using the TNBS method (Adler-Nissen, 1979) and antioxidant activity using ABTS radical scavenging activity assay. The result was expressed as the remaining activity in comparison to the undigested sample. In addition, cytoprotective effect of FBSH-III before and after simulated pepsin-pancreatin GI digestion was also determined as described in section 5.3.5.4.

5.3.7 Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA). Duncan's new multiple range tests was carried out for determination of the significant difference within the 95% confidence interval using SPSS 17.0 software (SPSS Inc, Chicago, IL, USA).

5.4 Results and discussion

5.4.1 Amino acid composition

After ultrafiltration, yield of FBSH-IV was the highest, followed by FBSH-III, FBSH-I and FBSH-II, respectively ($p<0.05$, Table 5.1). FBSH and ultrafiltrated fractions were rich in Lys, Glu/Gln, Gly, Pro, Ala, Asp/Asn, and Arg (Table 5.2). Amino acid profiles of FBSH and its fraction were slightly different. FBSH-III

contained higher amounts of Asp/Asn than other fractions. In addition, FBSH-IV contained higher amount of Val than other fractions ($p<0.05$) but was not different from FBSH-III ($p>0.05$). FBSH contained the highest Cys and essential amino acids. FBSH-III showed higher amounts of negatively charged amino acids than those of FBSH. Amounts of total hydrophobic, positively charged and aromatic amino acids of all fractions were comparable ($p>0.05$). Antioxidant activity of peptides depended on their amino acid compositions. Aromatic amino acids (Tyr, His, Trp and Phe) can make reactive oxygen species (ROS) stable through direct electron transfer, while maintaining their stability via resonance structures (Qian, Jung, and kim, 2008; Rajapakse, Mendis, Jung, Je, and Kim, 2005b). In addition, Trp and Tyr contain the indolic and phenolic groups, respectively, which serve as hydrogen donors. Acidic (Asp and Glu) and basic AAs (His, Lys and Arg) amino acid with carboxyl and amino groups in the side chains are thought to play an important role in chelating metal ions (Saiga, Tanabe, and Nishimura, 2003; Zhang et al., 2010). Hydrophobic amino acids may increase the solubility of some peptides in the lipid system and thereby facilitating better interaction with the radical species (Chen, Muramoto, Yamauchi, and Nokihara,1996).

FBSH-III and FBSH-IV showed the highest surface hydrophobicity ($p<0.05$, Table 5.2). No significant difference was observed among other samples ($p>0.05$). The lower surface hydrophobicity of the larger peptides (FBSH, FBSH-I, and FBSH-II) could be due to size effect and/or steric hindrance. The localization of hydrophobic binding sites in the larger peptides could limit the accessibility of ANS. In contrast, FBSH-III and FBSH-IV contained higher amount of small peptides (Figure 5.1d-e), contributing to the full exposure of hydrophobic sites with ANS. In addition, it is

possible that smaller peptides contain high amount of hydrophobic amino acid residues than larger peptides. Tang et al. (2009) speculated that an increase in Ho of buckwheat protein hydrolysate during extensive hydrolysis may be attributed to the release of more hydrophobic peptides from the globulins or the albumin aggregates (e.g., the albumin aggregates).

5.4.2 Size exclusion chromatography

Figure 5.1a-e shows the Superdex peptide 10/300 GL chromatograms of FBSH and ultrafiltrated fractions. The major peaks 1 and 2 observed in all chromatograms indicated MW around 1481-7000 Da. The first peak area of FBSH-IV was the lowest, implying that high MW peptides were relatively lower than other fractions. In addition, the greater peak area of Peaks no. 3 and 4 with the Mw about 430-1481 Da was observed in FBSH-II, -III and -IV as compared to larger peptides (FBSH-I, Figure 5.1b-e). This confirmed that low MW peptides in FBSH-III and -IV were relatively higher than FBSH-I.

Table 5.1 Peptide content, protein content and peptide yield of FBSH and ultrafiltrated fractions.

Sample	α-Amino acid content (mg leucine equivalents/ g sample)	Protein content (g/g sample)	Total peptide (mg leucine equivalents)	Peptide yield (%)
FBSH	126.5±6.5[b]	0.392±0.04	4731	100
FBSH-I	117.7±10.0[b]	0.397±0.05	467	10
FBSH-II	122.3±3.3[b]	0.422±0.01	351	7
FBSH-III	95.1±2.8[a]	0.424±0.04	767	16
FBSH-IV	116.3±11.3[b]	0.366±0.04	1958	41

Note: Peptide yield of each fraction was calculated based on α-amino acid content of FBSH. Protein content was calculated based on total amino acid content.

Values are expressed as means ± S.D. (n = 2).

Table 5.2 Amino acid composition (g/100 g protein), and surface hydrophobicity (H_0) of FBSH and ultrafiltrated fractions.

Amino acid	FBSH	FBSH-I	FBSH-II	FBSH-III	FBSH-IV
Ala	8.60±0.06	8.65±0.07	8.68±0.05	9.00±0.25	8.70±0.26
Arg	7.19±0.44	7.49±0.27	7.69±0.42	6.95±0.55	7.89±0.66
Asp/Asn	7.11±0.35^{a}	7.30±0.01^{a}	7.37±0.15^{a}	8.61±0.50^{b}	7.30±0.72^{a}
Cys	4.69±0.52^{c}	2.24±0.31^{b}	1.41±0.04^{a}	2.23±0.19^{b}	1.26±0.14^{a}
Glu/Gln	12.01±0.06^{a}	12.42±0.03^{ab}	12.49±0.01^{ab}	12.76±0.11^{b}	12.48±0.43^{ab}
Gly	10.94±0.92	11.24±0.12	11.14±0.52	10.17±0.56	11.16±1.05
His	2.25±0.15	2.22±0.08	2.31±0.12	2.30±0.16	2.63±0.29
Ile	4.32±0.21	4.41±0.06	4.50±0.13	4.46±0.30	4.78±0.45
Leu	2.44±0.27	2.49±0.34	2.26±0.06	2.39±0.21	2.40±0.26
Lys	12.81±0.26	12.86±0.12	13.24±0.01	10.94±2.71	11.76±2.96
Met	2.22±0.09	2.26±0.12	2.33±0.12	2.38±0.15	2.48±0.28
Phe	3.44±0.20	3.49±0.01	3.54±0.10	3.53±0.30	3.93±0.37
Pro	10.43±0.05	10.76±1.22	10.96±0.79	10.75±0.38	10.52±1.12
Ser	2.83±0.22	3.09±0.18	3.04±0.08	3.55±0.42	3.13±0.55
Thr	2.79±0.16	2.88±0.02	2.92±0.02	3.39±0.31	3.05±0.44
Typ	0.87±0.11	0.93±0.13	0.88±0.02	1.03±0.10	0.83±0.09
Tyr	1.76±0.14	1.87±0.08	1.80±0.04	1.93±0.21	1.85±0.09
Val	3.31±0.09 ^{a}	3.38±0.13^{a}	3.45±0.14^{a}	3.65±0.22^{ab}	3.85±0.09^{b}
TAA	100.00	100.00	100.00	100.00	100.00
TTHP	53.02±1.65	51.73±0.02	50.94±0.63	51.50±0.97	51.77±0.14
PCAA	22.24±0.85	22.58±0.24	23.24±0.55	20.19±2.31	22.28±2.01
NCAA	19.12±0.41^{a}	19.72±0.02^{ab}	19.86±0.14^{ab}	21.37±0.61^{b}	19.78±1.15^{ab}
EAA	40.89±0.4^{b}	39.05±0.97^{ab}	38.63±0.78^{a}	38.22±0.89^{a}	38.82±0.44^{a}
AAA	6.07±0.23	6.29±0.06	6.22±0.15	6.49±0.60	6.61±0.55
H_0	5852±19.1^{a}	5914±83.4^{a}	5669±277.2^{a}	10785±7.1^{b}	11323±753.1^{b}

Note: TAA is total amino acids; TTHP is total hydrophobic amino acids-Ala, Val, Ile, Leu, Tyr, Phe, Trp, Pro, Met, and Cys; PCAA is positively charged amino acids- Arg, His, Lys. NCAA is negatively charged amino acids-Asx and Gsx. AAA is aromatic amino acids-Phe, Trp, and Tyr. H_0 is surface hydrophobicity. Different letters indicate means with significant differences ($P<0.05$). Values are expressed as means ± S.D. (n=2).

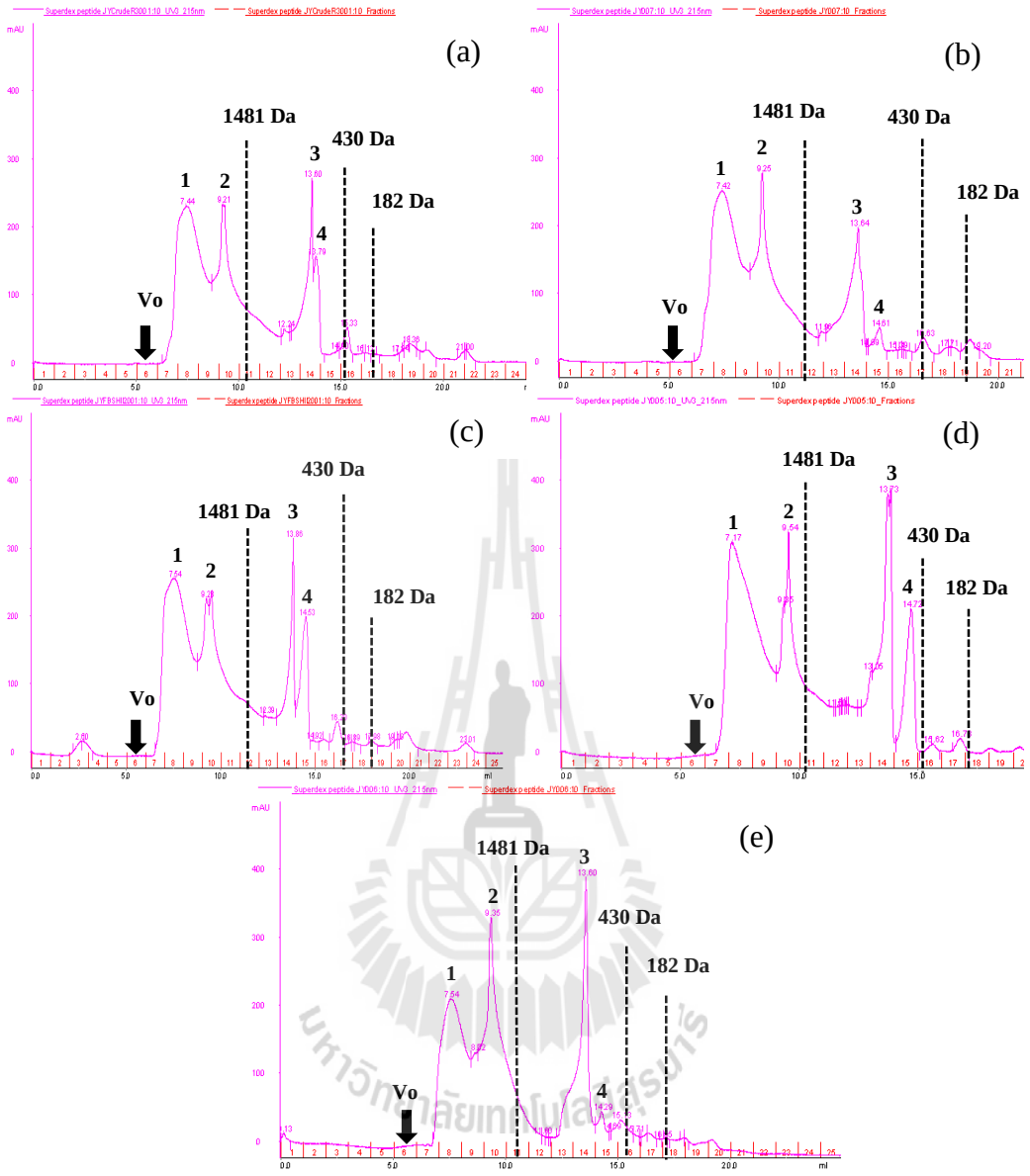

Figure 5.1 Size exclusion chromatograms of FBSH (a), FBSH-I (b), FBSH-II (c), FBSH-III (d) and FBSH-IV (e). Sample was loaded at 1.6 mg leucine equivalents/ml.

5.4.3 Antioxidant activity of ultrafiltrated fractions

ABTS radical scavenging activity of all fractions increased with concentration (Figure 5.2a). FBSH-III had the highest ABTS radical scavenging activity at all tested concentrations ($p<0.05$). Although FBSH contained the highest Cys (Table 5.2), which has been reported to display ABTS radical scavenging activity, this fraction exhibited lower ABTS radical scavenging activity than did FBSH-III. In addition, the amount of Try, Trp and His in all fractions were comparable (Table 5.2), indicating that total amino acid composition is not the only factor involved in the ABTS radical scavenging activity, peptide size and amino acid sequences might play a more crucial role in the ABTS radical scavenging activity.

Samaranayaka, Kitts, and Li-Chan (2010) reported that even though ultrafiltrated Pacific hake protein hydrolysate with peptides size of 1-3 kDa had the greatest ABTS radical scavenging activity, the total amino acid compositions of Pacific hake protein hydrolysate and UF fractions were relatively similar. In addition, oligopeptides with sizes <1400 Da were found to be major contributors to the ABTS radical scavenging activity with little dependence on peptide composition (Cheung, Cheung, Tan, and Li-Chan, 2012).

Evaluation of antioxidant activity using ferric reducing power assay (FRAP) is based on electron transfer mechanism of antioxidants (Raghavan, Kristinsson, and Leeuwenburgh, 2008). It has been reported that there is a direct correlation between antioxidant activities and reducing power of peptide (Li et al., 2010; Tang et al., 2012). As shown in Figure 5.2b, FRAP value of all fractions increased in linear fashion with peptide concentration ($p<0.05$). FBSH-III also showed the highest FRAP value in all tested concentrations followed by FBSH-IV ($p<0.05$). Amount of amino

acids which are known to be able to donate electron (Try, Trp and His) of all fractions were similar, except for Cys (Table 5.2), however MW distribution of all fractions was different (Figure 5.1a-e). FBSH-III and -IV likely contained high proportion of smaller peptides than other fractions and could contribute to high FRAP value. Smaller peptides are expected to expose more amino acid residue side chains which can donate electron and become more accessible by Fe^{3+}-TPTZ complex. However, a decrease of reducing power of FBSH-IV indicated that smaller peptides did not exhibit reducing power. This suggested that the reducing power of protein hydrolysates is dependent not only on the peptide size, but also to a highly influence on their sequences. Generally, peptides with low MW are thought to possess stronger antioxidant properties. He, Girgih, Malomo, Ju, and Aluko (2013) reported that only rapeseed peptides with MW <1 kDa obtained from ultrafiltration possessed ferric reducing power. High reducing power of small peptides may be contributed to hydrophilicity and electron donating ability (Liu et al., 2011). Wu, Chen, and Shiau (2003) reported that reducing power of mackerel hydrolysates was dependent on their MW distribution and MW of approximately 1400 Da showed stronger reducing power than that of 900- and 200-Da peptides. Bamdad, Wu, and Chen (2011) reported that the highest reducing power of hordein hydrolysate prepared from flavourzyme at 0.5 h digestion was observed around ~ 2 to 5 kDa. Porcine plasma protein hydrolysate with the highest reducing power was reported to be fractionated by ultrafiltration with MW < 3 kDa (Liu, Kong, Xiong, and Xia, 2010). In addition, Di Bernardini et al. (2012) reported that FRAP value of the three fractions, namely unfractionated sarcoplasmic proteins hydrolysate, the 10-kDa ultrafiltrated hydrolysate, and 3-kDa ultrafiltrated hydrolysate were comparable.

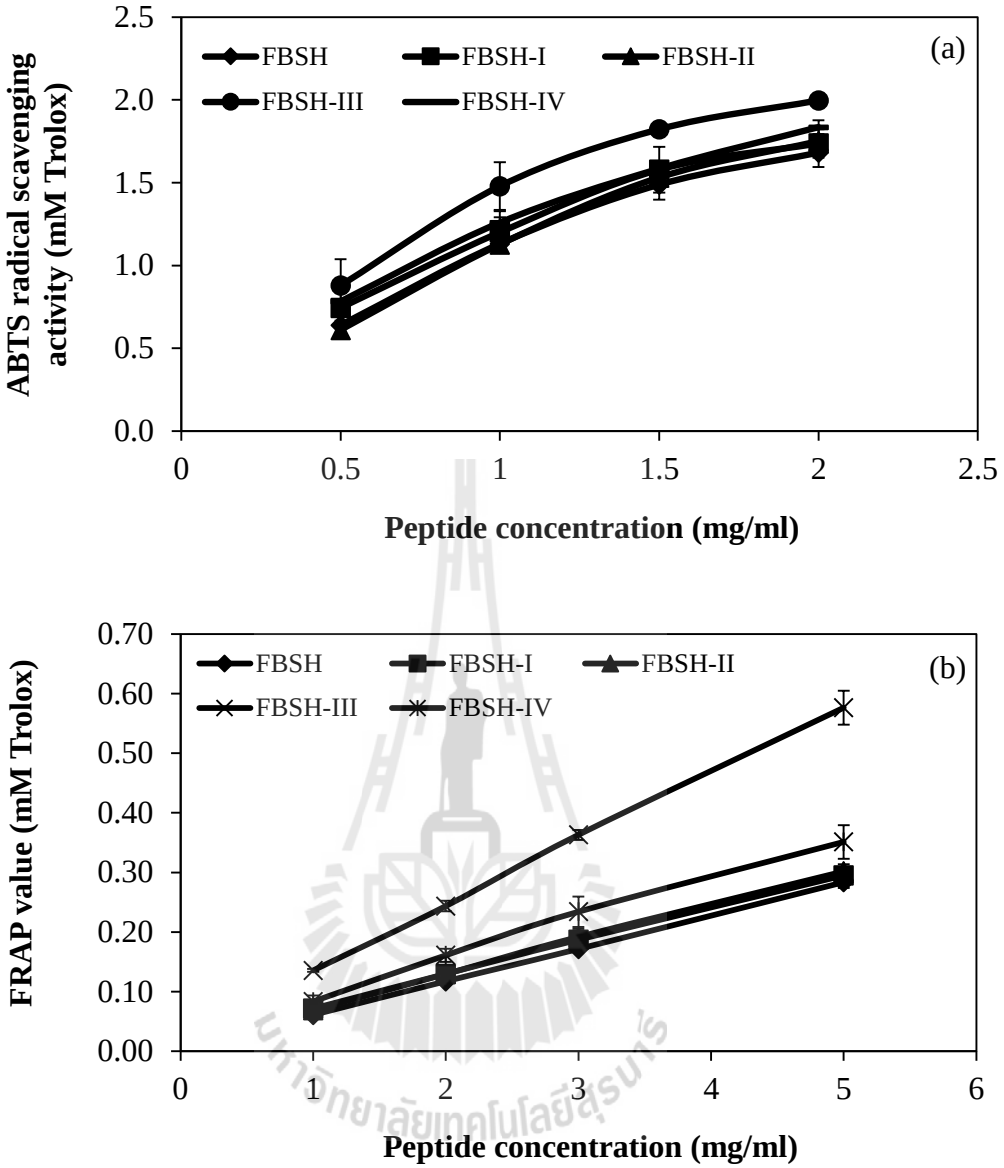

Figure 5.2 Changes in ABTS radical scavenging activity (a), and ferric reducing anti-oxidant power (b) of FBSH and its fractions at various peptide concentrations. Values are expressed as means ± S.D. (n=2).

All fractions exhibited dose-dependent metal chelating activity (Figure 5.3a). At 5 mg/ml, the highest metal chelating activity was observed in FBSH-III (0.45±0.02 mM EDTA) followed by FBSH-IV (0.24±0.01 mM EDTA). FBSH-III contained higher amount of Asp than other fractions (Table 5.2) which might contribute to higher metal chelating activity. Peptides with molecular weight around 3-5 kDa had the highest metal chelating activity followed by peptides with lower molecular weight (<3 kDa) and larger molecular weight (>5 kDa) (Hwang, Shyu, Wang, and Hsu, 2010). In addition, specific peptide structures and amino acid side chain residues may influence the ability to chelate metal ions (Decker, Crum, and Calvert, 1992). Generally, His is frequently observed in sequences of peptide ion chelator, especially the presence of His at their N-terminal positions results in strong metal chelating activity, since N in the imidazole ring often participates in coordination with metals (Chen, Muramoto, Yamauchi, Fujimoto, and Nokihara, 1998; Rajapakse et al., 2005b). The carboxyl and amino groups of the side chain amino acids, such as Glu, Asp, His, Lys and Arg, can interact with metal ions and inactivate their pro-oxidant activity (Saiga et al., 2003; Wu et al., 2003). Farvin, Baron, Nielsen, Otte, and Jacobsen (2010) reported that the low molecular weight (LMW) fractions of yoghurt peptides (3-10 and <3 kDa) contained a higher proportion of peptides with acidic amino acid residues at the C-terminus, whereas the high molecular weight (HMW) fractions of yoghurt peptides (>30 and 10-30 kDa) contained more peptides with a hydrophobic residue at the C-terminus. LMW fractions had higher metal chelating activity than those HMW fractions. The authors suggested that the presence of a C-terminal acidic amino acid residue, leading to a peptide with two carboxylic acids with high affinity towards cations. Moreover, the authors also suggested that if such

peptides are located close to each other it might result in a Fe^{2+} ion chelating ability by four carboxylic acid groups, like in EDTA. This might explained why 3-10 kDa and <3 kDa peptides were better metal chelators than >30 kDa and 10-30 kDa peptides.

Hydroxyl radical ($^{\bullet}OH$) is highly reactive compared to other reactive oxygen species (ROS). Generation of hydroxyl radicals in biological system can lead to damages of amino acids, proteins, DNA, and membrane components (Je, Lee, Lee, and Ahn, 2009). FBSH-III exhibited the highest hydroxyl radical scavenging activity followed by FBSH-IV ($p<0.05$, Figures 5.3b). The low molecular weight peptide (1096 Da) from duck processing by-products showed high hydroxyl radical scavenging activity (Lee et al., 2010). In addition, type of amino acids, such as His, Met and Cys specific peptide structures and amino acid sequences are responsible to hydroxyl radical scavenging activity (Hernández-Ledesma, Dávalos, Bartolomé, and Amigo, 2005). Peptides with a Tyr residue at their C-termini (Lys-Tyr, Arg-Tyr, and Tyr-Tyr) of Royal jelly protein hydrolysates showed strong hydroxyl radical scavenging activity (Guo, Kouzuma, and Yonekura, 2009).

FBSH-III showed the highest antioxidant activities based on various assays applied (Figures 5.2 and 5.3a-b). FBSH-I and FBSH-II, which were the retentate of the 30-kDa, and retentate of the 5-kDa membrane respectively, exhibited lower antioxidant activities than FBSH-III and FBSH-IV retentate of the 1-kDa membrane, and the permeate of the 1-kDa membrane, respectively. Total amino acid composition of FBSH and ultrafiltrated fractions were similar, indicating that total amino acid composition is not the only factor involved in antioxidant activity, peptide size and amino acid sequences might play a more crucial role in the antioxidant activity.

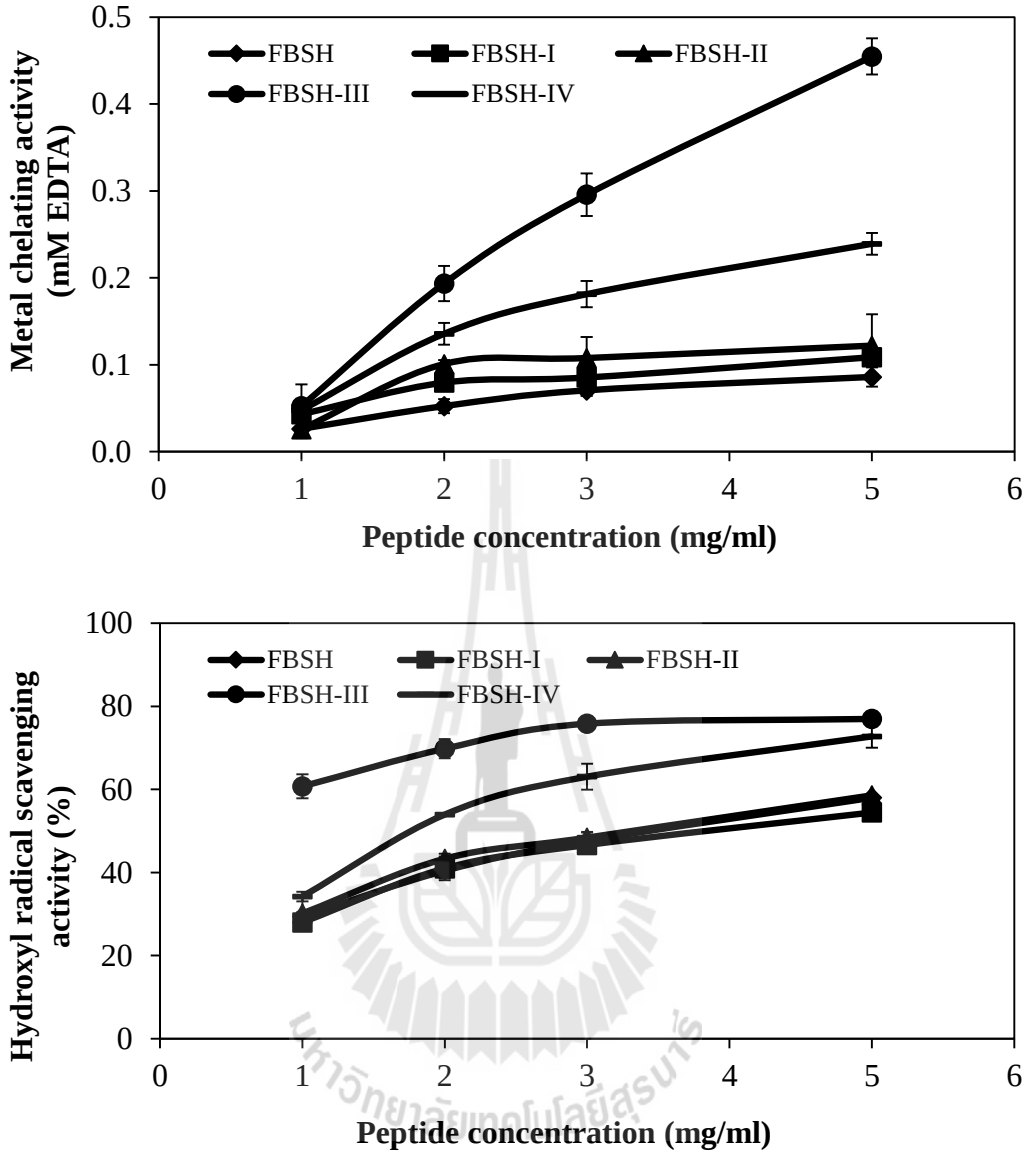

Figure 5.3 Changes in metal chelating activity (a), and hydroxyl radical scavenging activity (b) of FBSH and its fractions at various peptide concentrations. Values are expressed as means ± S.D. (n=2).

5.4.4 Protective effect of ultrafiltrated FBSH

Cytotoxicity of FBSH and ultrafiltrated fractions was tested on Caco-2 cell line to determine non-cytotoxic concentration. The results showed that FBSH and ultrafiltrated fractions did not show any cytotoxic effects on Caco-2 cell line at peptide concentrations of 25-200 µg/ml (Appendix B, Figure 1B). Therefore, range of peptide concentrations at 25-200 µg/ml was used for further experiments. When cells were treated with various concentrations of TBHP (0.2-1.0 mM) for 4 h, they were killed in a dose-dependent manner (Appendix B, Figure 2B). About 63% cell death was observed after cells were exposed to 0.5 mM TBHP. This concentration was, thus, chosen for the investigation of the protective effect.

Decomposition of TBHP to alkoxyl and peroxyl radicals will accelerate lipid peroxidation chain reactions and induce cell toxicity by damaging DNA (Aherne and O'Brien, 2000; Lapshina, Zavodnik, Labieniec, Rekawiecka, and Bryszewska, 2005). This decomposition is aided by metal ions and their complexes leading to the generation of different ROS (Guidarelli, Cattabeni, and Cantoni, 1997). FBSH-III exhibited the highest cytoprotective effect (Figure 5.4a). When cells were treated with TBHP, cells were killed about 63% (Figure 5.4b). However, cell viability was increased with concentration of FBSH-III and enhanced viability of TBHP-induced cytotoxicity up to 82% was observed at peptide concentration of 200 µg/ml (Figure 5.4b). FBSH-III showing high surface hydrophobicity (Table 5.2) may efficiently penetrate into lipid bilayer of Caco-2 cells and scavenge with lipid-derived radicals at cell membranes. This result suggested that FBSH-III decreased TBHP-induced cytotoxicity. However, FBSH-III showed lower cytoprotective effect than did 25 µg/ml α-tocopherol.

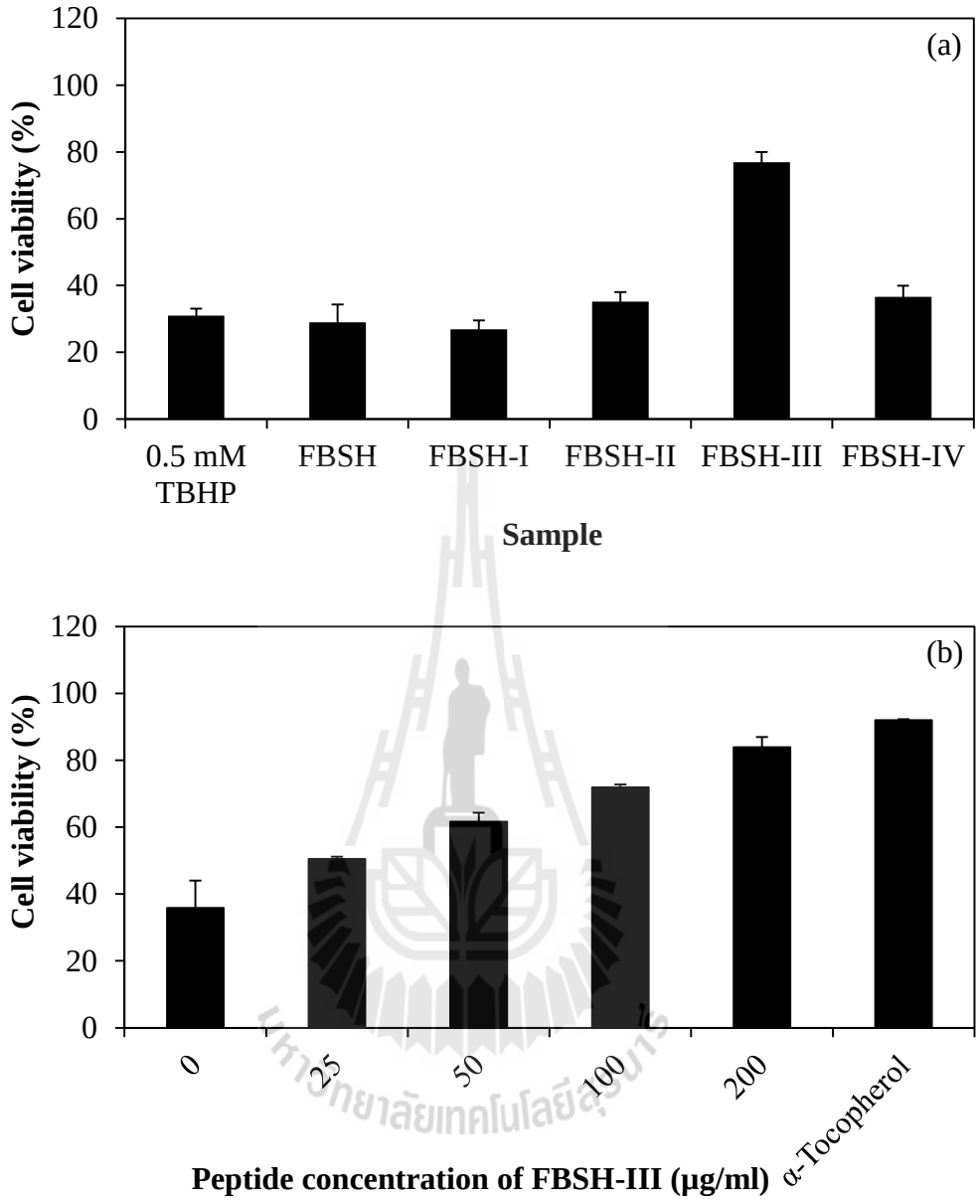

Figure 5.4 Cytoprotective effects of FBSH and its ultrafiltrated fractions at peptide concentration of 200 μg/ml (a), and the effect of FBSH-III at different peptide concentrations on TBHP-induced cytotoxicity to Caco-2 cells (b). Values are expressed as means ± S.D. (n=5).

5.4.5 Effect of FBSH-III on TBHP-induced LDH leakage

Damage to cells caused by a variety of xenobiotics frequently results in an early alteration of cell membrane permeability, leading to the leakage of cytoplasmic enzymes, like lactate dehydrogenase (LDH) (Ponsoda, Jover, Castell, and Gomez-Lechon, 1991). Therefore, LDH leakage was used as an indicator for a rapid evaluation of cell membrane integrity and cytotoxicity induced by TBHP (Alía et al., 2005). FBSH-III pretreated cells did not show LDH leakage as compared to the control (cell without treatment) (Appendix C, Figure 1C). This indicated that FBSH-III did not show any cytotoxicity effects on Caco-2 cell at peptide concentrations of 25-200 µg/ml. TBHP-treated cell showed higher amounts of LDH leakage as compared to the untreated cell (Figure 5.5, $p<0.05$), suggesting TBHP-induced cytotoxicity. Cells pretreated with FBSH-III at concentration as low as 25 µg/ml exhibited less LDH leakage. The extent of cell leakage as judged by LDH activity reduced when FBSH-III increased to 50 µg/ml and was comparable to α-tocopherol at 25 µg/ml. Further increase peptide content up to 200 µg/ml did not show greater protective effect. FBSH-III at a concentration of 50 µg/ml was able to reduce cytotoxicity induced by TBHP.

5.4.6 Effect of FBSH-III on cellular ROS

DCFH-DA is widely used to measure oxidative stress in cells due to the high sensitivity of this fluorescence-based assay (Bonini, Rota, Tomasi, and Mason, 2006). DCFH-DA penetrates into the cells and is hydrolyzed to DCFH by intracellular esterases. The presence of ROS can oxidize DCFH to form DCF, a fluorescent product. Evaluation of the intracellular ROS, such as H_2O_2, superoxide anion ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and hydroxyl radicals ($^{\bullet}OH$), using fluorescent DCF can be used

as an index of the overall oxidative stress within cells (Wang, and Joseph, 1999). The fluorescent intensity of the cells treated with 0.3 mM TBHP increased as compared to the control (cell without TBHP) (Figure 5.6). Ascorbic acid (25 µg/ml) was used as a positive control, which reduced the DCF fluorescent intensity about 54% compared to cell with 0.3 mM TBHP. The pretreatment with FBSH-III peptide at a concentration of 25 µg/ml reduced the DCF fluorescent intensity approximately 16% compared to cells without peptide. An increase in peptide concentration up to 100 µg/ml did not further reduce DCF fluorescent intensity. The result suggested that FBSH-III peptide at 50 µg/ml was able to inhibit intracellular TBHP-induced ROS production. However, its efficacy was less than ascorbic acid.

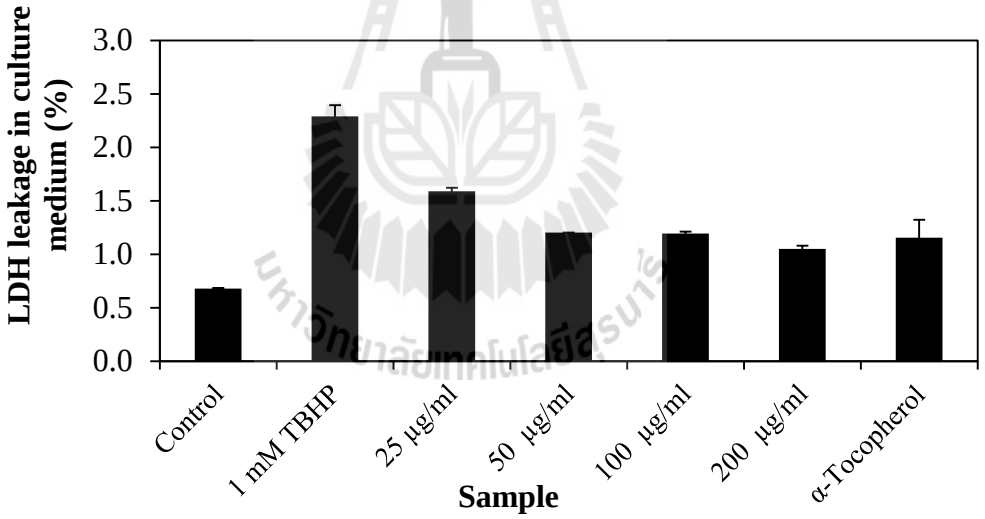

Figure 5.5 Changes in LDH activity in culture medium pre-treated with FBSH-III at various peptide concentrations for 12 h and followed by the treatment of 1 mM TBHP for 3 h. Control was cultured without FBSH-III and TBHP. α-Tocopherol was used at 25 µg/ml. Values are expressed as means ± S. D. (n=4).

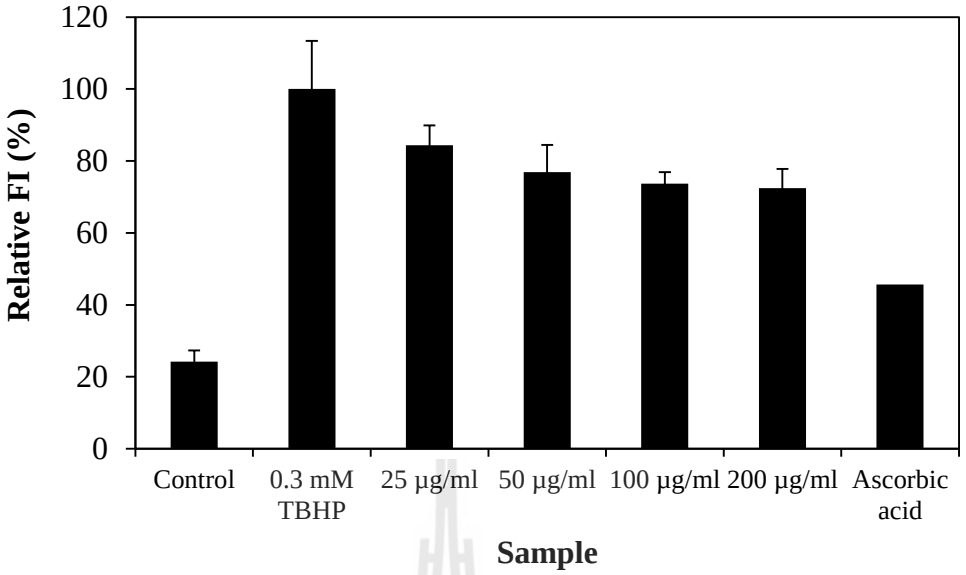

Figure 5.6 Cellular radical scavenging activity of FBSH-III. Caco 2 cells were labeled with 10 µM fluorescence dye, DCFH-DA, and treated with different concentrations of FBSH-III (25, 50, 100 and 200 µg/ml) for 12 h. Ascorbic acid of 25 µg/ml was used as a positive control. Fluorescence intensities of DCF due to oxidation of DCFH by cellular ROS (generated by 0.3 mM TBHP) were detected at E_x= 485 nm and E_m= 528 nm, and expressed as relative fluorescence intensity (FI). Values are expressed as means ± S.D. (n=4-5).

5.4.7 Stability of FBSH-III

5.4.7.1 Temperature and pH stability

ABTS radical-scavenging activity increased after thermal treatments of FBSH-III (Figure 5.7a). After heating at 121°C, ABTS radical-scavenging activities of FBSH-III increased by 20% compared to the sample without heat treatment. These results indicated that peptides in FBSH-III were stable toward thermal treatment. Therefore, incorporation of FBSH-III in thermally-processed foods would be plausible without loss of its antioxidant activity.

After subjected to different pH conditions for 1 h, FBSH-III could retain their ABTS radical-scavenging activities compared to the untreated sample at pH 7.4 (Figure 5.7b). It indicated that peptides in FBSH-III were stable at both acidic and alkaline pH. Nalinanon et al. (2011) also found that ABTS radical-scavenging activity of the antioxidant peptide from the muscle of ornate threadfin bream hydrolysate remained constant over the pH range of 1–10. In addition, peptides with the short chains and amino acids in protein hydrolysates were not much affected by charge modification governed by pH changes (Klompong et al., 2008). Therefore, FBSH-III could be applicable at a wide pH range.

5.4.7.2 *In vitro* pepsin–pancreatin simulated GI digestion

Antioxidant properties of peptide could be further modified upon gastrointestinal (GI) digestion. To illustrate such an effect, FBSH-III was incubated with pepsin for 3 h and subsequently digested by pancreatin for 4 h. Pepsin digestion did not increase α-amino acid content of FBSH-III (Figure 5.8a). However, α-amino acid content of FBSH-III increased after pancreatin digestion. An increase in *α*-amino content suggested that peptide bonds of FBSH-III were cleaved only by pancreatin.

Pepsin shows high specificity toward aromatic amino acids: Phe, Tyr, and Trp, and Leu and Glu at the carboxyl side of a peptide bond (Simpson, 2000), while pancreatin contains many enzymes, including the endopeptidases trypsin, α-chymotrypsin, and elastase and the exopeptidases carboxypeptidases A and B with broad specificity (Young, Nau, Pasco, and Mine, 2011), leading to higher proteolytic digestion of FBSH-III. Our results exhibited similar pattern with You, Zhao, Regenstein, and Ren (2010) who reported that fewer peptide bonds were broken by pepsin than by pancreatin digestion.

Pepsin digestion did not alter ABTS radical-scavenging activity of FBSH-III (Figure 5.8b). After pancreatin digestion, ABTS radical-scavenging activity increased by 23.7% as compared to the undigested counterpart ($p<0.05$). During pancreatin digestion, peptides with smaller masses were generated to a greater extent (Figure 5.8a), which could lead to modifications of the antioxidant peptide sequences. Smaller peptides with specific sequence for antioxidant activity were, therefore, formed. This result indicated that some peptides with stronger antioxidant activities were produced during the simulated gastrointestinal digestion. Nalinanon et al. (2011) also reported that pepsin digestion had no effect on ABTS radical-scavenging activity of fish muscle hydrolysate, but ABTS radical-scavenging activity of peptides was increased by about 7-12% after pancreatin digestion.

For TBHP-induced cytotoxicity assay, both, FBSH-III and after *in vitro* pepsin-pancreatin digestion protected cells from TBHP-induced cytotoxicity at peptide concentration of 50 µg/ml compared to control (cell without TBHP) (Figure. 5.8b), and the cell viability of both fractions were comparable ($p>0.05$). These results indicated that FBSH-III digests still performed cytoprotection in the similar extent to

FBSH-III. Therefore, FBSH-III is expected to have cellular antioxidant activity after ingestion in both stomach and small intestine.

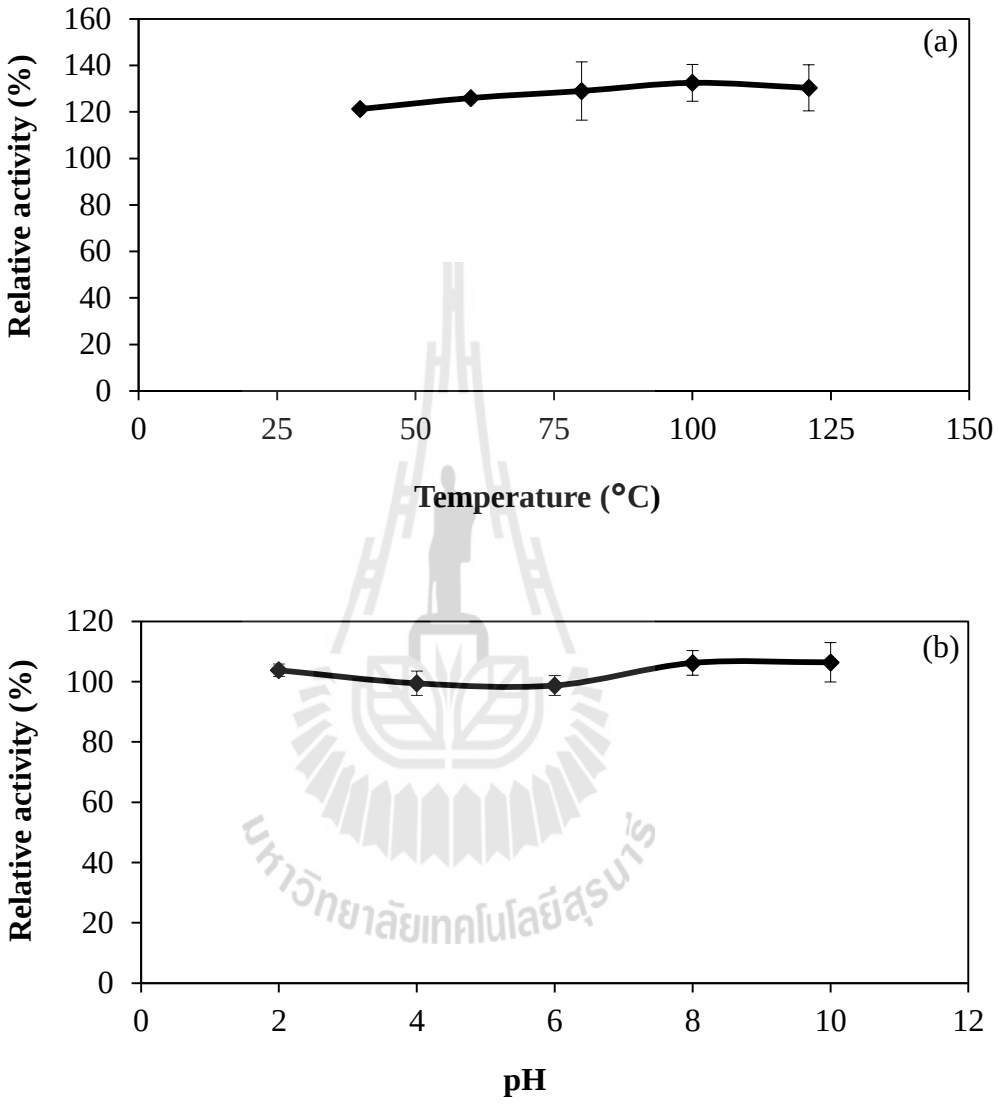

Figure 5.7 Effects of temperature (a), and pH (b) on of antioxidant activity of FBSH-III. Values are expressed as means ± S.D. (n=2).

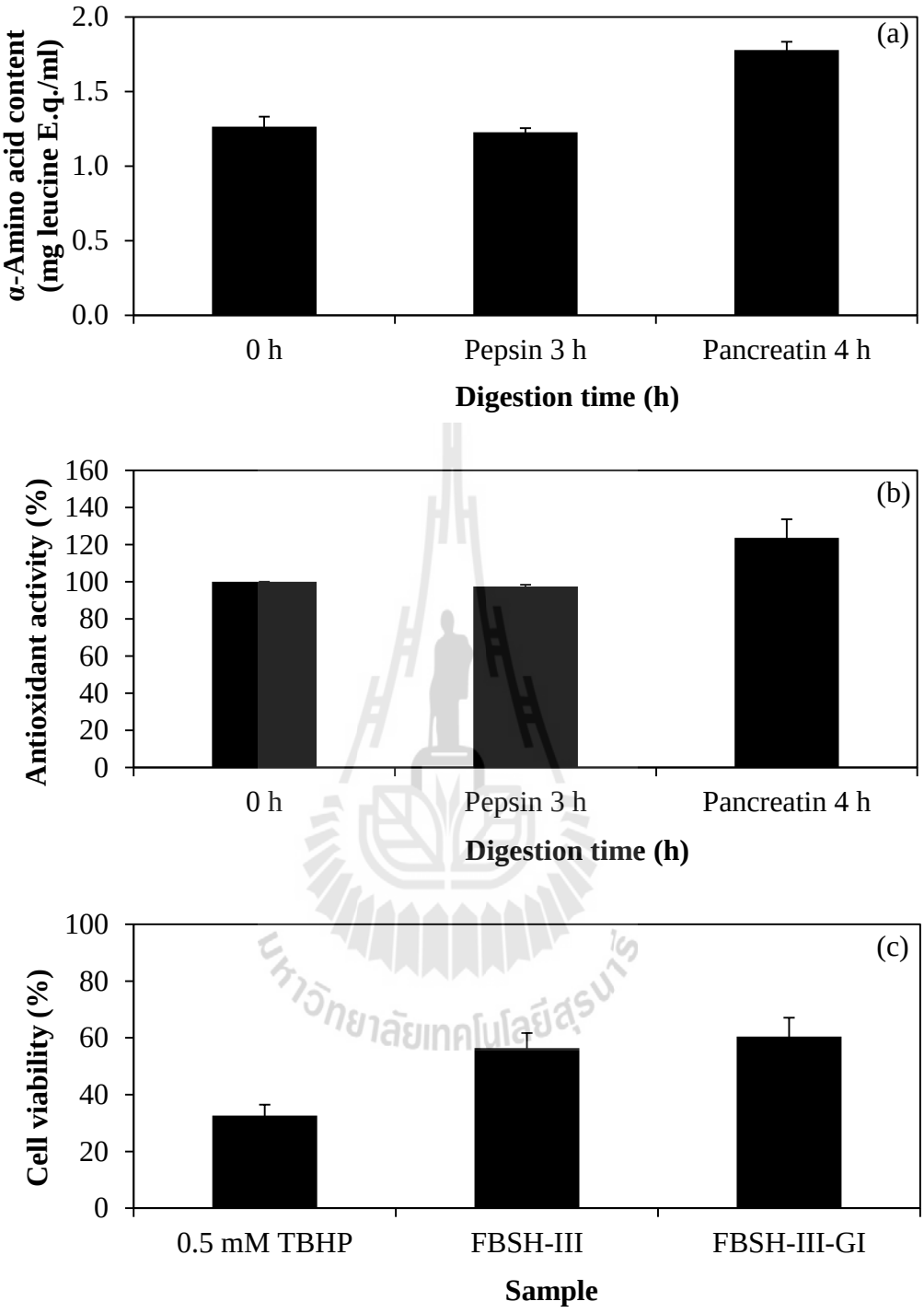

Figure 5.8 α-Amino acid content (TNBS method) (a) and antioxidant activities of FBSH-III after *in vitro* pepsin-pancreatin simulated GI digestion measured by ABTS radical-scavenging activity assay (b), and TBHP-induced cytotoxicity using MTT assay (c).

5.5 Conclusion

Ultrafiltrated fractions had higher antioxidant activities compared to the parent hydrolysate (FBSH). FBSH-III, retentate of 1-kDa-membrane obtained from the 5-kDa permeate (1-5 kDa), exhibited the highest antioxidant activities and cytoprotective effect, depending on peptide concentrations. FBSH-III showed potential to be used as a food additive and/or neutraceutical peptides due to antioxidative properties, and its stability under high temperature, a broad range of pH and GI digestion.

5.6 References

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolyzates by trinitrobenzenesulfonic acid. **Journal of Agricultural and Food Chemistry.** 27: 1256-62.

Aherne, S. A. and O'Brien, N. M. (2000). Mechanism of protection by the flavonoids, quercetin and rutin, against tert-butylhydroperoxide- and menadione-induced DNA single strand breaks in Caco-2 cells. **Free Radical Biology and Medicine.** 29: 507-514.

Alía, M., Ramos, S., Mateos, R., Bravo, L., and Goya, L. (2005). Response of the antioxidant defense system to t-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). **Journal of Biochemical and Molecular Toxicology.** 19: 119-128.

AOAC (2000). **Official methods of analysis** (17th ed.). Gaithersburg, MD, USA: Association of Official Chemists.

Bamdad, F., Wu, J., and Chen, L. (2011). Effects of enzymatic hydrolysis on molecular

structure and antioxidant activity of barley hordein. **Journal of Cereal Science.** 54: 20-28.

Bonini, M. G., Rota, C., Tomasi, A., and Mason, R. P. (2006). The oxidation of 2',7'-dichlorofluorescin to reactive oxygen species: a self-fulfilling prophesy? **Free Radical Biology and Medicine.** 40: 968-75.

Brand-Williams, W., Cuvelier, M. E., and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. **Lebensmittel-Wissenschaft and Technologie.** 28: 25-30.

Calabrese, E., La Seta, F., Buccellato, A., Virdone, R., Pallotta, N., Corazziari, E.,and Cottone, M. (2005). Crohn's disease: A comparative prospective study of transabdominal ultrasonography, small intestine contrast ultrasonography, and small bowel enema. **Inflammatory Bowel Diseases.** 11: 139-145.

Carrasco-Castilla, J., Hernández-Álvarez, A. J., Jiménez-Martínez, C., Jacinto-Hernández, C., Alaiz, M., Girón-Calle, Vioque, J and Dávila-Ortiz, G. (2012). Antioxidant and metal chelating activities of peptide fractions from phaseolin and bean protein hydrolysates. **Food Chemistry** 135: 1789-1795.

Cheung, I. W. Y., Cheung, L. K. Y., Tan, N. Y., and Li-Chan, E. C. Y. (2012). The role of molecular size in antioxidant activity of peptide fractions from Pacific hake (*Merluccius productus*) hydrolysates. **Food Chemistry.** 134: 1297-1306.

Chen, M. and Li, B. (2012). The effect of molecular weights on the survivability of casein-derived antioxidant peptides after the simulated gastrointestinal digestion. **Innovative Food Science and Emerging Technologies.** 16: 341-348.

Chen, H. M., Muramoto, K., Yamauchi, F., Fujimoto, K., and 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.

Chen, H. M., Muramoto, K., Yamauchi, F., and Nokihara, K. (1996). Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. **Journal of Agricultural and Food Chemistry.** 44: 2619-2623.

Cinq-Mars, C. D., Hu, C., Kitts, D. D., and Li-Chan, E. C. Y. (2008). Investigations into inhibitor type and mode, simulated gastrointestinal digestion, and cell transport of the angiotensin I-Converting enzyme-inhibitory peptides in pacific hake (*Merluccius productus*) fillet hydrolysate. **Journal of Agricultural and Food Chemistry.** 56: 410-419.

Cornelli, U. (2009). Antioxidant use in nutraceuticals. **Clinics in Dermatology.** 27: 175-194.

Decker, E. A., Crum, A. D., and Calvert, J. T. (1992). Differences in the antioxidant mechanism of carnosine in the presence of copper and iron. **Journal of Agricultural and Food Chemistry.** 40: 756-759.

Di Bernardini, R., Mullen, A. M., Bolton, D., Kerry, J., O'Neill, E., and Hayes, M. (2012). Assessment of the angiotensin-I-converting enzyme (ACE-I) inhibitory and antioxidant activities of hydrolysates of bovine brisket sarcoplasmic proteins produced by papain and characterisation of associated bioactive peptidic fractions. **Meat Science.** 90: 226-235.

Ekelund, K., Osth, K., Pahlstorp, C., Bjork, E., Ulvenlund, S., and Johansson, F. (2005). Correlation between epithelial toxicity and surfactant structure as derived from the effects of polyethyleneoxide surfactants on Caco-2 cell

monolayers and pig nasal mucosa**. Journal of Pharmaceutical Sciences.** 94: 730-744.

Farvin, K.H.S., Baron, C.P., Nielsen, N.S., Otte, J., and Jacobsen, C. (2010). Antioxidant activity of yoghurt peptides: Part 2 – Characterisation of peptide fractions. **Food Chemistry.** 123: 1090-1097.

Guidarelli, A., Cattabeni, F., and Cantoni, A. (1997). Alternative mechanisms for hydroperoxide-induced DNA single strand breaks. **Free Radical Research.** 26: 537-547.

Guo, H., Kouzuma, Y., and Yonekura, M. (2009). Structures and properties of antioxidative peptides derived from royal jelly protein. **Food Chemistry.** 113: 238-245.

Halliwell, B. (1996). Antioxidants in human health and disease. **Annual Review of Nutrition.** 16: 33-50.

He, R., Girgih, A. T., Malomo, S. A., Ju, X., and Aluko, R. E. (2013). Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. **Journal of Functional Foods.** 5: 219-227.

Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., and Amigo, L. (2005). Preparation of Antioxidant Enzymatic Hydrolysates from α-Lactalbumin and ß-Lactoglobulin. Identification of Active Peptides by HPLC-MS/MS. **Journal of Agricultural and Food Chemistry.** 53: 588-593.

Hidalgo, I. J., Raub, T. J., and Borchardt, R. T. (1989). Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. **Gastroenterology.** 96: 736-749.

Hwang, J. Y., Shyu, Y. S., Wang, Y. T., and Hsu, C. K. (2010). Antioxidative properties

of protein hydrolysate from defatted peanut kernels treated with esperase. **LWT - Food Science and Technology**. 43: 285-290.

Je, J. Y., Lee, K. H., Lee, M. H., and Ahn, C. B. (2009). Antioxidant and antihypertensive protein hydrolysates produced from tuna liver by enzymatic hydrolysis. **Food Research International**. 42: 1266-1272.

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

Klompong, V., Benjakul, S., Kantachote, D., Hayes, K. D., and Shahidi, F. (2008). Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from Alcalase and Flavourzyme. **International Journal of Food Science and Technology.** 43: 1019-1026.

Ktari, N., Jridi, M., Bkhairia, I., Sayari, N., Ben Salah, R., and Nasri, M. (2012). Functionalities and antioxidant properties of protein hydrolysates from muscle of zebra blenny (*Salaria basilisca*) obtained with different crude protease extracts. **Food Research International.** 49: 747-756.

Lapshina, E. A., Zavodnik, I. B., Labieniec, M., Rekawiecka, K., and Bryszewska, M. (2005). Cytotoxic and genotoxic effects of *tert*-butyl hydroperoxide on Chinese hamster B14 cells. **Mutation Research.** 583: 189-97.

Lee, S. J., Kim, E. K., Hwang, J. W., Oh, H. J., Cheong, S. H., Moon, S. H., Jeon, B. T., Lee, S. M., and Park, P. J. (2010). Purification and characterisation of an antioxidative peptide from enzymatic hydrolysates of duck processing by-products. **Food Chemistry.** 123: 216-220.

Li, H. M., Hu, X. I. N., Guo, P., Fu, P., Xu, L. I., and Zhang, X. Z. (2010). Antioxidant

properties and possible mode of action of corn protein peptides and zein peptides. **Journal of Food Biochemistry.** 34: 44-60.

Lima, C. F., Andrade, P. B., Seabra, R. M., Fernandes-Ferreira, M., and Pereira-Wilson, C. (2005). The drinking of a Salvia officinalis infusion improves liver antioxidant status in mice and rats. **Journal of Ethnopharmacology.** 97: 383-389.

Liu, Q., Kong, B., Xiong, Y. L., and Xia, X. (2010). Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. **Food Chemistry.** 118: 403-410.

Liu, J. H., Tian, Y. G., Wang, Y., Nie, S. P., Xie, M. Y., Zhu, S. (2011). Characterization and in vitro antioxidation of papain hydrolysate from black-bone silky fowl (*Gallus gallus domesticus Brisson*) muscle and its fractions. **Food Research International.** 44: 133-138.

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

Nalinanon, S., Benjakul, S., Kishimura, H., and Shahidi, F. (2011). Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. **Food Chemistry.** 124: 1354-1362.

Pappenheimer, J. R. and Volpp, K. (1992). Transmucosal impedance of small intestine-correlation with transport of sugars and amino acids. **American Journal of Physiology.** 263: C480-493.

Ponsoda, X., Jover, R., Castell, J., and Gomez-Lechon, M. (1991). Measurement of intracellular LDH activity in 96-well cultures: A rapid and automated assay for

cytotoxicity studies. **Journal of tissue culture methods.** 13: 21-24.

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

Raghavan, S., Kristinsson, H. G., and Leeuwenburgh, C. (2008). Radical Scavenging and Reducing Ability of Tilapia (*Oreochromis niloticus*) Protein Hydrolysates. **Journal of Agricultural and Food Chemistry.** 56: 10359-10367.

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

Rajapakse, N., Mendis, E., Jung, W. K., Je, J. Y., and Kim, S. K. (2005b). Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. **Food Research International**. 38: 175-182.

Saiga, A., Tanabe, S., and Nishimura, T. (2003). Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. **Journal of Agricultural and Food Chemistry.** 51: 3661-3667.

Samaranayaka, A. G. P., Kitts, D. D., and Li-Chan, E. C. Y. (2010). Antioxidative and angiotensin-I-converting enzyme inhibitory potential of a Pacific Hake (*Merluccius productus*) fish protein hydrolysate subjected to simulated gastrointestinal digestion and Caco-2 cell permeation. **Journal of Agricultural and Food Chemistry**. 58: 1535-1542.

Sheih, I. C., Fang, T. J., and Wu, T. K. (2009). Isolation and characterisation of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from the algae

protein waste. **Food Chemistry.** 115: 279-284.

Simmonds, N. J., and Rampton, D. S. (1993). Inflammatory bowel disease-a radical view. **Gut.** 34: 865-868.

Simpson, B. K. (2000). Digestive proteinases from marine animals. In N. M., Haard, and B. K. Simpson (Eds.). **Seafood enzymes: Utilization and influence on postharvest seafood quality** (pp. 531-540). New York: Marcel Dekker.

Tang, C. H., Peng, J., Zhen, D. W., and Chen, Z. (2009). Physicochemical and antioxidant properties of buckwheat (*Fagopyrum esculentum Moench*) protein hydrolysates. **Food Chemistry**. 115: 672-678.

Tang, X., Wu, Q., Le, G., Wang, J., Yin, K., and Shi, Y. (2012). Structural and antioxidant modification of wheat peptides modified by the heat and Lipid peroxidation product malondialdehyde. **Journal of Food Science.** 77: H16-H22.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. **The International Journal of Biochemistry and Cell Biology**. 39: 44-84.

Valko, M., Morris, H., and Cronin, M. T. D. (2005). Metals, toxicity and oxidative stress. **Current Medicinal Chemistry**. 12: 1161-1208.

Wang, H. and Joseph, J. A. (1999). Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. **Free Radical Biology and Medicine**. 27: 612-616.

Wiriyaphan, C., Chitsomboon, B., and Yongsawadigul, J. (2012). Antioxidant activity of protein hydrolysates derived from threadfin bream surimi byproducts. **Food Chemistry.** 132: 104-111.

Wu, H. C., Chen, H. M., and Shiau, C. Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). **Food Research International.** 36: 949-957.

You, L., Zhao, M., Regenstein, J. M., and Ren, J. (2010). Changes in the antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysates during a simulated gastrointestinal digestion. **Food Chemistry.** 120: 810-816.

Young, D., Nau, F., Pasco, M., and Mine, Y. (2011). Identification of hen egg yolk-derived phosvitin phosphopeptides and their effects on gene expression profiling against oxidative stress-induced Caco-2 Cells. **Journal of Agricultural and Food Chemistry.** 59: 9207-9218.

Zhang, J. H., Zhang, H., Wang, L., Guo, X. N., Wang, X. G., and Yao, H. Y. (2010). Isolation and identification of antioxidative peptides from rice endosperm protein enzymatic hydrolysate by consecutive chromatography and MALDI-TOF/TOF MS/MS. **Food Chemistry.** 119: 226-234.

CHAPTER VI

SUMMARY

Frame, bone, and skin (FBS) is a potential source for production of protein hydrolysate with antioxidant properties. Antioxidant activity of FBS and refiner discharge (RD) protein hydrolysates based on chemical assays, activities showed a similar pattern, but did not correlate with cell-based assay. Cell-based assay showed comparable cytoprotective effect of FBS and RD hydrolysates, but variations on chemical assays, 2, 2'-azinobis (3-ethyl-benzothiazoline-6-sulfonate) (ABTS) radical scavenging, ferric reducing antioxidant power (FRAP) and β-carotene bleaching, were noticed. In addition, antioxidant activity does not necessarily increase with DH. Specific amino acid composition in the peptide sequences is a more important factor manifesting antioxidant activity of protein hydrolysate. Compared to commercial proteinases, *Virgibacillus* sp. SK33 proteinase can be used in production of protein hydrolysate with antioxidant property.

Further fractionation of FBS hydrolysate prepared from *Virgibacillus* sp. SK33 proteinase using anion exchange and size exclusion columns could improve antioxidant activity. The fraction from size exclusion chromatography with the smallest mass peptides showed distinct ABTS radical scavenging activity and FRAP value, while the moderate mass peptides showed distinct hydroxyl radical scavenging activity and metal chelating activity. In addition, the synthetic FLGSFLYEYSR peptide obtained from pooled de novo peptide sequencing of the fraction B3 obtained

from size exclusion chromatography appeared to be of important antioxidant peptides, which scavenged both synthetic radicals and cellular ROS.

Fractionation of FBS hydrolysate by ultrafiltration using membranes with various molecular weight cut-off values yielded FBSH-III, which was a retentate of 1-kDa-membrane obtained from the 5-kDa permeate (1-5 kDa). FBSH-III showed higher antioxidant activities than the parent hydrolysate, and protected Caco-2 cell from oxidative damage-induced by *tert*-butylhydroperoxide (TBHP). Total amino acid composition of FBSH and ultrafiltrated fractions were similar, indicating that total amino acid composition is not the only factor involved in antioxidant activity. Peptide size, hydrophobicity and amino acid sequences might play a more crucial role in the antioxidant activity. FBSH-III showed potential to be used as a food additive and/or neutraceutical peptides due to antioxidative properties, cytoprotective effect and its stability under high temperature, a broad range of pH and Gastrointestinal (GI) digestion.

APPENDIX

Supporting materials for Chapter III and V

Appendix A

Cytotoxicity of FBS or RD hydrolysates on HepG2 cell

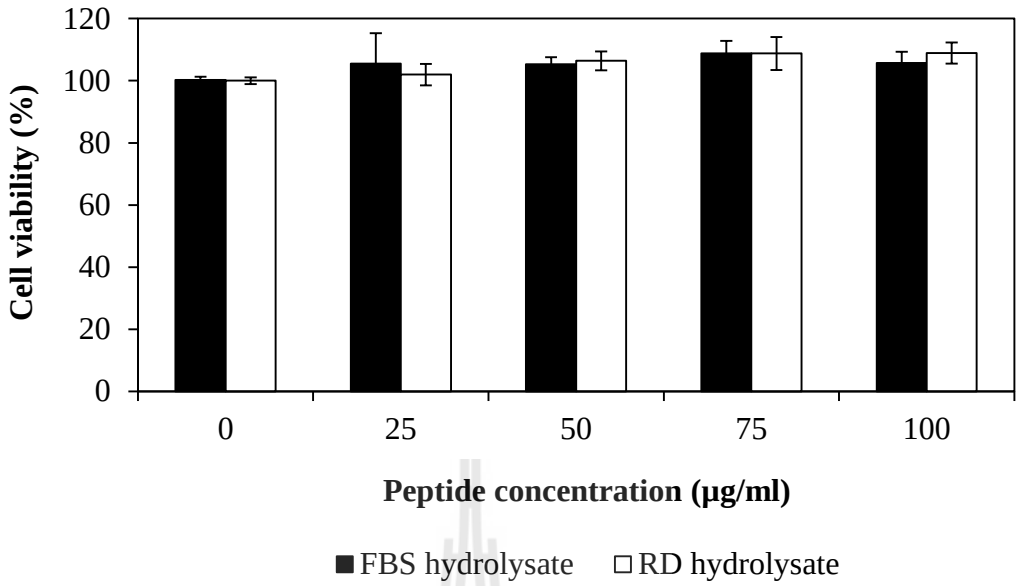

Figure 1A Viability of HepG2 cells after incubation with FBS or RD hydrolysates prepared from *Virgibacillus* sp. SK33 proteinase at various peptide concentrations. Values are expressed as means ± S.D. (n= 4).

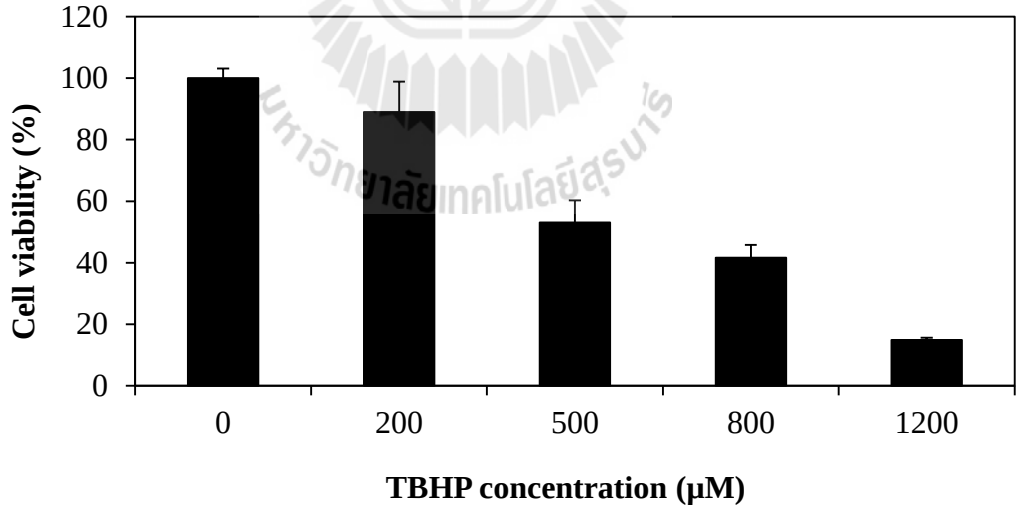

Figure 2A *Tert*-butylhydroperoxide (TBHP)-induced cytotoxicity on HepG2 cells as various concentrations (0-1200 µM).

Values are expressed as means ± S.D. (n=4).

Appendix B

Cytotoxicity of FBSH and ultrafiltrated fractions on Caco-2 cell

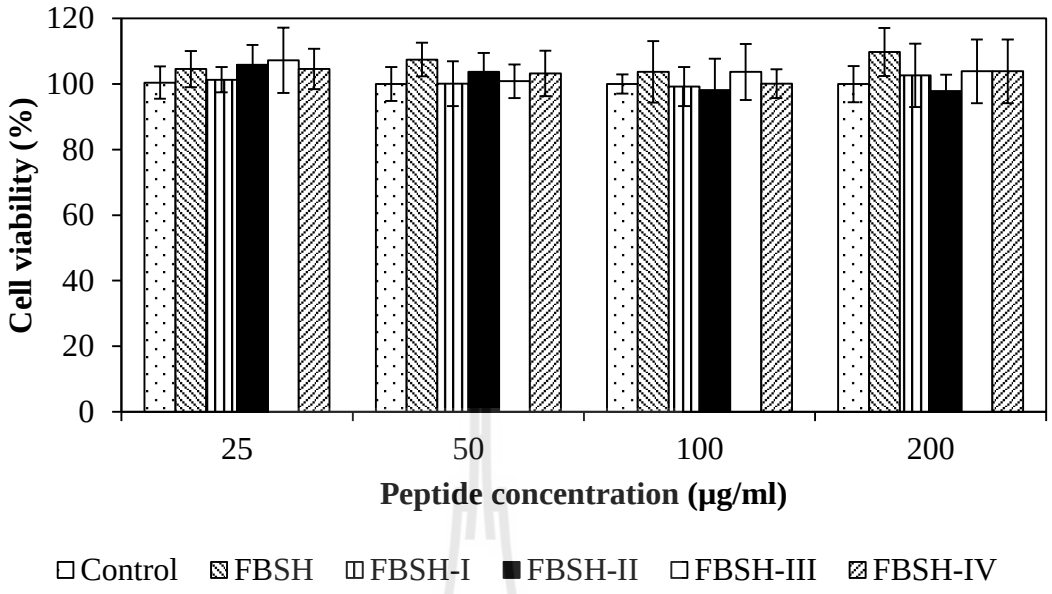

Figure 1B Viability of Caco-2 cells after incubation with FBSH and ultrafiltred fraction derived from *Virgibacillus* sp. SK33 proteinase at various peptide concentrations. Values are expressed as means ± S.D. (n = 2).

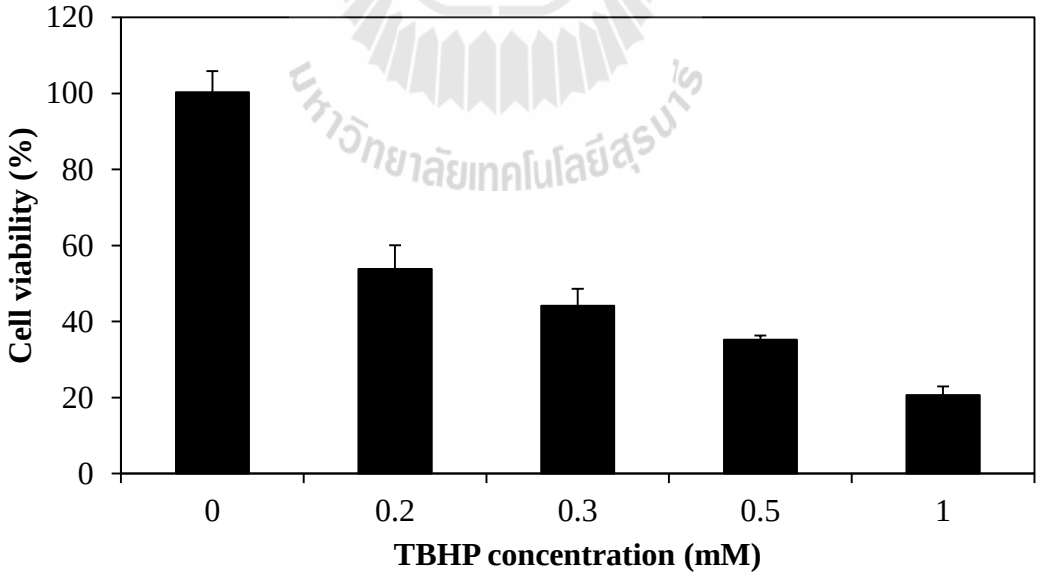

Figure 2B *Tert*-butylhydroperoxide (TBHP)-induced cytotoxicity on Caco 2 cells as various concentrations (0.2-1.0 mM).

Values are expressed as means ± S.D. (n=7).

Appendix C

LDH leakage in Caco-2 cell

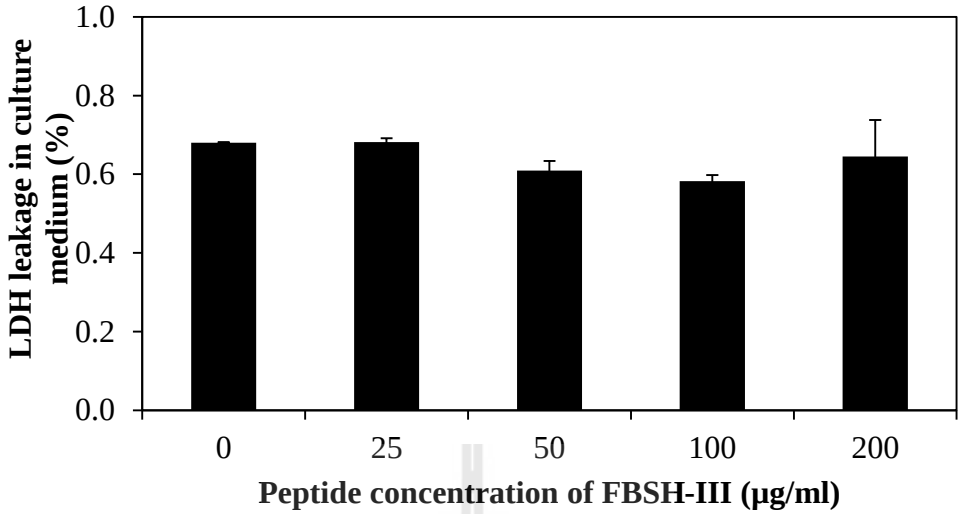

Figure 1C Changes in LDH activity in culture medium pre-treated with FBSH-III at various peptide concentrations for 12 h. Values are expressed as means ± S.D. (n = 4).

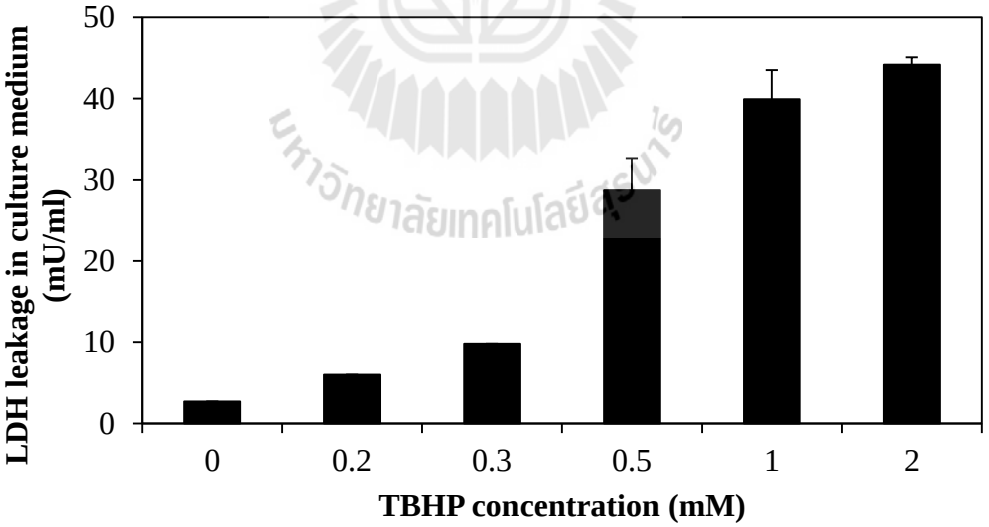

Figure 2C *Tert*-butylhydroperoxide (TBHP)-induced LDH leakage on Caco-2 cells as various concentrations (0-2 mM). Values are expressed as means ± S.D. (n = 4).

Appendix D

Degree of hydrolysis (DH) and ABTS radical scavenging activity of FBSHs

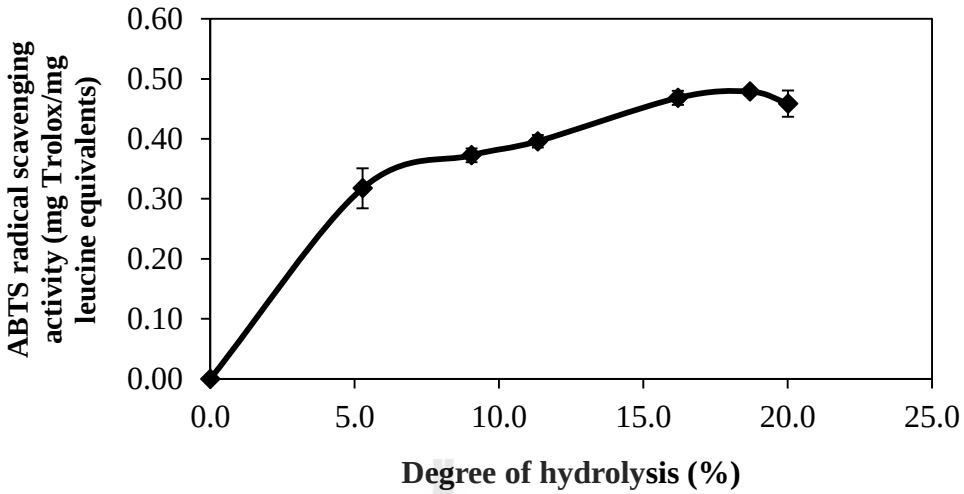

Figure 1D Effect of different DH on ABTS radical scavenging activity of FBSHs prepared from *Virgibacillus* sp. SK33. Values are expressed as means ± S.D. (n = 2).

Appendix E

LC-MS/MS of FBSH-III

The amino acid sequences of active fraction were determined using an ESI-QUAD-TOF (micro TOF-QTM Basic System, Bruker, Germany) with electrospray ionization. The peptides were separated in an Easy-nL C II column (C18-AQ, 3 µm, 100Å, 75 µm id x 100 mm). Eluent A consisted of 0.1% formic acid in water, and eluent B consisted of 100% acetonitrile with 0.1% formic acid. The elution was performed using a 30-min linear gradient of 0 to 30% of eluent B at a flow rate of 300 nl/min. Peptide fragment mass spectra were acquired in data-dependent AutoMS mode with a scan range of 350-3000 m/z, 3 averages, and up to 5 precursor ions selected from the MS scan 100-3000 m/z. Peptide peaks were detected and deconvoluted automatically using Data Analysis version 4.0 SP 5. Mass lists in the form of Mascot generic files were created automatically and used as the input for Mascot MS/MS Ions searches of the National Center for Biotechnology Information nonredundant (NCBI nr) database (http://www.matrixscience.com). Default search parameters used were the following: Enzyme = pepsinA, max. missed cleavages = 1; fixed modifications = carbamidomethyl (C); variable modifications = oxidation (M); peptide tolerance ±0.5 Da; MS/MS tolerance ±0.5 Da; peptide charge = 2+, 3+ and 4+; instrument = ESI–QUAD-TOF.

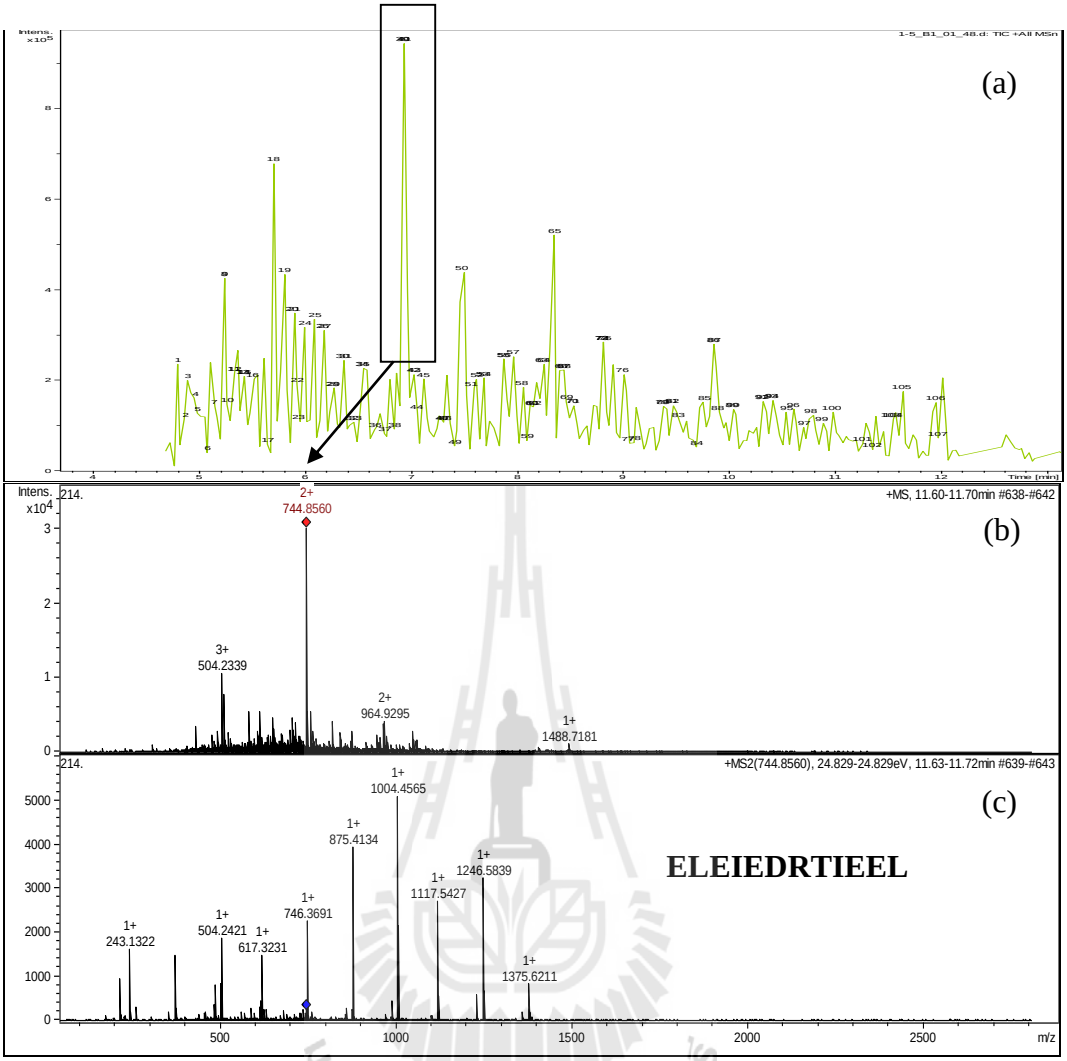

Figure 1E UV chromatogram of the FBSH-III (a), Mass spectrum of the selected chromatographic peak in Figure 5.8a (b). Tandem mass spectrum of ion m/z 744.8560 (c).

Table 1E Peptides identified by LC coupled to ESI-QUAD-TOF of FBSH-III

No	Mr(expt)	Mr(calc)	Peptide sequences
1	1275.5349	1275.6605	MGTGSLAKDRAGL
2	957.4729	957.5131	TGEIGLQQL
3	1487.6972	1487.7355	ELEIEDRTIEEL
4	1896.8919	1896.8755	QGAWAEWPVGEQGQNQL
5	1101.5069	1101.5720	AVRWQSGLW
6	1055.5225	1055.5836	REALGQRGGL
7	1869.7830	1869.9545	TAIGQNVAGGNASISGGAVNL
8	1283.5722	1283.6357	QAVALGPNDTADL
9	1194.5187	1194.6469	HGQGLSAARVSL
10	1303.5690	1303.6190	DALQDCAAASRAL
11	1827.7969	1827.9227	DADTAESIAHRFVRQL
12	1411.6492	1411.8147	TTGIVANRINGLAL
13	2381.0746	2381.1672	QKDDCVLAISYSGESEEIVAIL
14	1272.5856	1272.6674	ITGAAGGVGSAATQL
15	1104.5710	1104.5274	VAMIENNPAF
16	1911.8914	1911.8785	QGSVQPSCPEHSSLASWL
17	1111.5206	1111.6390	WGKPITGGAIL
18	2196.9608	2196.9883	TAQYDDRSSGNQQGDTATGLL
19	1793.8528	1793.9233	GSVIDAAVIAKYCDASVL
20	1145.6147	1145.6519	VAIGIPLSMVF
21	1088.5433	1088.5350	SIGTGSTPADAL
22	1199.5861	1199.6986	KGVLANSKDRL
23	1121.5102	1121.6081	RAGLITDTYL
24	1109.4698	1109.4336	DPLGSDDGGMF
25	1245.5670	1245.6088	EIEEENKDKL
26	2025.9222	2026.1058	DKLREKPNIEVENKTTL
27	1741.7968	1741.8920	ECIVAPEISAEAREIL
28	1641.6755	1641.8461	SGIDGEIIIGEQLADL
29	2137.9342	2138.0015	GVSEDDVVDGLAVGNAYTASSL
30	1849.8637	1849.9646	RQAAEQGIAPAQNALAEL
31	1566.7252	1566.8042	KIAEGKDAGKEFQF
32	1952.8536	1953.0948	WTRIDPVKFGPNIARAL
33	1203.6119	1203.5560	VADEQWTLGW
34	1956.8588	1957.0003	EAATAEQAKADLDAKIAEL
35	1679.7901	1680.0298	GAISDKSLIVPIAKRL

Table 1E (Continued)

No	Mr(expt)	Mr(calc)	Peptide sequences
36	1727.8142	1727.9417	IINNNIEEKEEKKK
37	3029.3680	3029.4777	QGGPNCPYSERYTATYRSSSGPAAKLRL
38	2135.0083	2135.1409	SLRQAIGIVMGANIGTTVTSF
39	2151.0066	2151.0062	WAVAAVALPGSGAEGDGGWYAF
40	1310.6167	1310.6103	GPAGPAEDQTQQL
41	1470.6731	1470.7467	KYGPDGQPVPQSAK
42	1681.7648	1681.8886	GEDIGALIKNGPKEEL
43	1137.4781	1137.5012	LNGIEDVECF
44	1698.8112	1698.8510	QNSQANTGRQARQQL
45	2156.9883	2156.9321	IGGEAGSAGSWSSCSTPGVDSVF
46	1517.6361	1517.8049	KLGDGTVESTRKDL
47	1841.8218	1841.9860	EIGPHRAKIEESRAHL
48	1684.6372	1684.8520	DVQVIEGVKEVDAGDL
49	1850.8743	1851.0102	AGAGAAIGAAGGVSELIIGSPL
50	1684.7896	1684.9222	LWVMGEQIIGGIKEL
51	897.4847	897.3981	HSEELHF
52	1668.6625	1668.8770	MRFINPPPSGRIGDL
53	886.4279	886.4396	EGGPTSGLGL
54	1620.7619	1620.9603	WVALTIVVNVVAPQL
55	1877.8978	1878.1163	AQAGANGILVARDIIKRL
56	1215.5521	1215.6863	GQLYTISPPKL
57	2420.0203	2420.1206	MAAASIERYFSEIDEIQDSVF
58	1935.8545	1936.0782	IPIAERSGQIVAIGDWVL
59	1511.7237	1511.6892	RYGEDGLAGEAVEF
60	1626.7569	1626.7534	MDNIPPFTHPMSQL
61	793.4337	793.3606	GELGAGSGF
62	1125.5307	1125.5125	PSDGCPQPAGVV
63	1497.6946	1497.7762	RPWGAGAVIGCLADL
64	897.3547	897.4994	SPAPVLMAL
65	2520.1254	2520.4414	ISVEQIKEEIKIELDKIGNIPL
66	2487.1375	2487.2805	AVEAVQVFGGSVVRYCHNARNVL
67	1715.8137	1715.8955	QISGAGWSVSGKSSPRL
68	1820.8501	1820.9884	IQGEPEITAGEVLAAVKP

Table 1E (Continued)

No	Mr(expt)	Mr(calc)	Peptide sequences
69	1872.8551	1872.8278	HESWDELREEVGYGAP
70	1978.8428	1979.0072	SRDLPTTQVSPPSGPVRGE
71	1942.8038	1942.9061	PGVGHIYEAAADAGSGIDPF
72	1055.5545	1055.5539	YPITYVSTL
73	1551.7301	1551.8270	RAPGAWQEANLARL
74	1835.8413	1835.8625	ENCRAAVPQGNFSPSIF
75	1602.7183	1602.7890	EGWLAGVTTKADGDAL
76	1710.6544	1710.7602	GNMPVPVGMFMNMSSL
77	1083.5610	1083.4907	EIAGSSCGTLF
78	1896.8919	1897.0428	AAMKRGALGGRGIERGGQL
79	3048.3461	3048.6243	INHYETKKSIELPENVPVSKPAGEKGGK
80	1341.6149	1341.6888	ESGAGSLEARPGVL
81	2572.1620	2572.2571	RHGESVWNKENRFTGWKDVDL
82	1156.5277	1156.5724	GSIGAESSHLSL
83	2550.1814	2550.2773	NDDIKSTSTSISTNGSPESKNKKL
84	1694.6845	1694.8475	VLSTTQESHPVGNDVL
85	1877.8984	1877.9703	SAAVTVLGTGIMGSGMARSL
86	2384.1156	2384.2621	EAGGVAVGDEVKINIEVEMVKAK
87	1857.8260	1857.8389	MREFSIQAAGAADMPYT
88	2172.9869	2173.2946	ELNNTKVTRKIYNIVKKL
89	2905.3998	2905.2900	KCNSCDALCATCNKISGKCITGNPGYEL
90	2135.0084	2135.0270	VSVPITTEQPSGTTSTEAPSF
91	3002.7331	3002.4841	SMTYSVIQQNGSAIPSEAMIFSSSARKL
92	1083.5550	1083.5924	ALEEPASRVL
93	2304.1076	2304.2698	RGMIGACNQLAITIGIVISYVL
94	2770.2933	2770.4753	IQGVIIDIGGSGVNDVSVIGVAEVNISF
95	1746.7451	1746.9208	ICSLRAGKCIMRHIF
96	2533.1182	2533.2997	TDRERDTAHAIAGGLSNPEIAARL
97	3085.4539	3085.7737	RKIEVVPTHRKDRKRAIFGVSAQPANL
98	3695.0886	3694.9770	IVGKEYEHKGIARDGAKMVHAVANANVPKFTVVF
99	2941.3484	2941.4967	RGIKEDPDAKIVGISPGCEGENIERLF
100	1203.5547	1203.6169	IEMAASEKVNL
101	3310.4723	3310.7283	ADTPTKTGLAYIRRQRASGPSPDGNRVAWGL
102	3368.0354	3367.6951	LAKTAASHAGAGADMVAPSDMMDGRVWAIRKAL

BIOGRAPHY

Chompoonuch Wiriyaphan was born in September 5th, 1984 in NongKhai, Thailand. She studied for high school at Pack Sauy Pityakhom School (1998-2003). In 2007, she received the degree of Bachelor of Science in Food Technology with first class honor from Suranaree University of Technology, Nakhon Ratchasima. In 2008-2012, she received the Royal Golden Jubilee Scholarship from Thailand Research Fund to study for the degree of Doctor of Philosophy (Food Technology) at Suranaree University of Technology. During her graduate study, she obtained opportunities to present her research works including at RGJ-Ph.D. Congress XIV (Pattaya, Chonburi, April 5-7th 2013) with awarded "Outstanding poster presentation" and IFT annual meeting and Food Expo (Las Vegas, Nevada USA, June 25-28th , 2012). She also published her researched work under the title "Antioxidant activity of protein hydrolysates derived from threadfin bream surimi byproducts" in Journal Food Chemistry (volume 132, page 104–111) in 2012.