ฤทธิ์ต้านอนุมูลอิสระและยับยั้งการอักเสบของเปปไทด์จากหอยหวาน *Babylonia areolata*

นายคมสัน เตโชอภิวัฒนกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF PEPTIDE FROM SPOTTED BABYLON *Babylonia areolata*

Mr. Komson Techoaphiwatanakun

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2015

Thesis Title	ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF PEPTIDE FROM SPOTTED BABYLON *Babylonia areolata*
By	Mr. Komson Techoaphiwatanakun
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Aphichart Karnchanatat, Ph.D.
Thesis Co-Advisor	Ninnaj Chaitanawisuti, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Associate Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Nattaya Ngamrojanavanich, Ph.D.)

.....Thesis Advisor
(Assistant Professor Aphichart Karnchanatat, Ph.D.)

.....Thesis Co-Advisor
(Ninnaj Chaitanawisuti, Ph.D.)

.....Examiner
(Associate Professor Chanpen Chanchao, Ph.D.)

.....External Examiner
(Chantragan Phiphobmongkol, Ph.D.)

คมสัน เตโชอภิวัฒนกุล : ฤทธิ์ต้านอนุมูลอิสระและยับยั้งการอักเสบของเปปไทด์จากหอยหวาน *Babylonia areolata* (ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF PEPTIDE FROM SPOTTED BABYLON *Babylonia areolata*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.อภิชาติ กาญจนทัต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.นิลนาจ ชัยธนาวิสุทธิ์, 59 หน้า.

ระดับอนุมูลอิสระที่ไม่เหมาะสมถือว่าเป็นหนึ่งในสาเหตุหลักของโรคต่างๆ ในชีวิต เมื่ออนุมูลอิสระอยู่ในระดับที่มากเกินและสารต้านอนุมูลอิสระไม่เพียงพอจนเกิดภาวะความเครียดออกซิเดชันนำไปสู่ความเสียหายของเซลล์และเนื้อเยื่อซึ่งสามารถลดความเสียหายได้จากการบริโภคสารต้านอนุมูลอิสระที่เพิ่มขึ้น ในการศึกษาเปปไทด์ที่ได้จากหอยหวาน (*Babylonia areolata*) ถูกย่อยด้วยเอนไซม์โปรตีเอส 3 ชนิด (อัลคาเลส นิวเตรสและเฟลเวอร์ไซม์) และเปปไทด์ที่ได้จากการย่อยด้วยเฟลเวอร์ไซม์เป็นเวลา 3 ชั่วโมง แสดงค่าการยับยั้ง DPPH ABTS NO SOD Catalase และ GPx ได้ค่าดีที่สุดเมื่อเทียบกับการย่อยด้วยเอนไซม์อื่นๆ จากนั้นทำการแยกเปปไทด์ด้วยเทคนิคอัลตราฟิลเตรชัน และเทคนิคโครมาโตกราฟีแบบเจลฟิลเตรชันและพบว่า F3 มีการต้านอนุมูลอิสระดีที่สุด หลังจากนั้นนำ F3 มาทดสอบการยับยั้ง NO และ MTT ในเซลล์ RAW 264.7 ทำบริสุทธิ์โดยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงซึ่งแสดงผล 4 พีค และทั้งหมดถูกวิเคราะห์ด้วยเทคนิคแมสสเปกโตรเมตรี พบสายของเปปไทด์ 6 สาย

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5672188123 : MAJOR BIOTECHNOLOGY

KEYWORDS: ANTIOXIDANT / PEPTIDE / PROTEASE / SPOTTED BABYLON / ANTI-INFLAMMATORY

KOMSON TECHOAPHIWATANAKUN: ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF PEPTIDE FROM SPOTTED BABYLON *Babylonia areolata*. ADVISOR: ASST. PROF. APHICHART KARNCHANATAT, Ph.D., CO-ADVISOR: NINNAJ CHAITANAWISUTI, Ph.D., 59 pp.

Inappropriate free radical levels are considered to be one of the major causes of various syndromes in living systems. Oxidative stress, which occurs when free radicals and antioxidant levels are in excess and insufficient, respectively, leading to cell and tissue damage, can be alleviated in some circumstances by the consumption of additional antioxidants. In this study, peptide derived from spotted babylon (*Babylonia areolata*) was hydrolysed using three kinds of proteases (alcalase, neutrase and flavozyme), and the flavozyme hydrolysate obtained by 3-h hydrolysis exhibited the highest DPPH, ABTS, NO, SOD, Catalase and GPx activities assay compared to other hydrolysates. By using ultrafiltration and gel filtration chromatography, the F3 fraction from the flavozyme hydrolysate of spotted Babylon was highest antioxidant activity. After that, F3 fraction from gel filtration chromatography was tested in RAW 264.7 cells by inhibition of NO production assay and MTT assay, and the F3 fraction was purified using HPLC technique. The chromatogram showed the profile which contained 4 main peaks. These 4 fractions will analyzed using mass spectrometry which was obtained 6 sequence in 4 peak.

Field of Study: Biotechnology
Academic Year: 2015

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my thesis advisor, Assistant Professor Dr. Aphichart Karnchanatat for his invaluable help and constant encouragement throughout the course of this research. I am most grateful to Dr. Nilnaj Chaitanawisuti for his teaching, advice and all support.

I express special thanks to my thesis committee: Associate Professor Dr. Nattaya Ngamrojanavanich, Associate Professor Dr. Chanpen Chanchao and Dr. Chantragan Phiphobmongkol for their suggestion and guidance.

I would like to thank teachers, researchers and officer at program in Biotechnology, Faculty of Science, and Institute of Biotechnology and Genetic Engineering, Chulalongkorn University.

Finally, I most gratefully acknowledge my parents and my friends for all their support throughout the period of this research.

CONTENTS

Page

Page

Page

LIST OF TABLE

Page

LIST OF FIGURE

Page

LIST OF ABBREVIATIONS

Abs	Absorbance
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
BSA	Bovine serum albumin
CO2	Carbon dioxide
DMSO	Dimethylsulfoxide
DPPH•	2,2-diphenyl-1-picrylhydrazyl radical
et al.	and others
eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
GPX	Glutathione peroxidase
GSH	Glutathione
h	Hour
H	hydrogen
H_2O	water
H_2O_2	Hydrogen peroxide
IC_{50}	Median inhibitory concentration, 50% maximum inhibition
iNOS	Inducible nitric oxide synthase
kDa	Kilodalton
LPS	Lipopolysaccharide
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-

	diphenyltetrazolium bromide
MW	Molecular weight
NaCl	Sodium chloride
NED	N-(1-naphthyl) ethylene diamine
nm	Nanometer
nNOS	Neuron nitric oxide synthase
NO•	Nitric oxide
O_2	oxygen
O_2•-	Superoxide anion
O.D.	Optical Density
PBS	Phosphate buffered saline
PGE2	Prostaglandin E2
Rpm	Round per minute
TNF-μ	Tumor necrosis factor alpha
UV	Ultraviolet
α	Alpha
β	Beta
℃	Degree Celsius
γ	Gamma
g	Microgram
μl	Microliter
μM	Micromolar
/	Per
%	Percentage
:	Ratio

CHAPTER I

INTRODUCTION

Nowadays, humans are exposed to elevated levels of free radicals from external sources such as UV rays, pollution, stress and poor nutrition. Free radical appears to play a major role and a cause of many diseases such as inflammation, cancer, ageing, heart attack, diabetes, dermatitis and other health complications. However, antioxidants are compounds that can be used to inhibit the free radicals. The compounds help prevent free radical damage by donation of electron. Normally, the antioxidants can be derived from the diet, such as vegetables and meat. The body is a substance that can inhibit and control free radical including antioxidant such as ceruloplasmin, transferrin, superoxide, dismutase, catalase, glutathione peroxidase and others substance.

In addition, the free radical damages endothelial cell and increases vascular permeability. Then, it is stimulating adhesion of neutrophil on endothelial cell, and motivated endothelial cell create up superoxide. Moreover, the free radical inhibits antiprotease which destructs extracellular matrix and cell tissue by enzyme from the lysosome. Therefore, the pharmaceutical industry is giving more attention to the new classes of protein drugs with the stability and bioavailability of small molecules. Peptide drugs, especially the short synthetic, fewer side-effects, low toxicity and long-acting ones, are quickly increasing in the global market. Inflammation is a protective immunovascular response that involves immune cells, blood vessels, and molecular mediators. The inflammation signs are pain, heat, redness, swelling, and loss of function. There is the need to consider new sources of drug to cure this effect. During inflammation, macrophages cells release chemical mediators such as nitric oxide and prostaglandins E2. These substances built from leukocytes by enzymes Cycloxigenase-2 (COX-2) and Inducible Nitric oxide synthase (iNOS). Nitric oxide and prostaglandins E2 Will be induced to create up a large number of cytokine and endotoxin. Nitric oxide is a chemical compound with chemical formula of NO which has an important role in the disease of inflammation. Then, it is an effective molecule in many biological systems such as immunological, neuronal,

cardiovascular, tissues, blood circulatory system and others. Then, the effect of prostaglandin E2 (PGE2) renders vasodilation and Increased vascular permeability.

Protein hydrolysis is a product of the protein digestion by digesting polypeptide chains into short-peptide chains which have antioxidant activity (Taherzadeh and Karimi 2007, Samaranayaka and Li-Chan 2011, Pokora, Eckert *et al*. 2013). There are 2 methods for the preparation of protein hydrolysate; acid-base extraction and enzymatic hydrolysis. This study will focus on enzymatic hydrolysis (Kim, Lee et al. 2009). The advantages of enzyme hydrolysis are the high conversion yield, nontoxic, biodegradable, high specificity and utilization of soft conditions. Recently, active peptides are the interesting sources of drug development (Moure, Cruz et al. 2001). So, this study will focus on new sources of protein hydrolysate from marine spotted babylon.

Spotted babylon (*Babylonia areolata*) (Chaitanawisuti and Kritsanapuntu 1999), belonging to the family Buccinidae, genus Babylonia, is an important gastropod consumption in Thailand. Spotted babylon has a high nutrition which contains 18.78% of protein, 17 amino acids (glutamic acid arginine and alanine up to 80.18% of the total free amino acid contents), fat 2.86%, carbohydrate 5.18%, ash 5.27%, and moisture 67.91%. meat and intestines of spotted babylon includes 17 amino acids. Spotted babylon could be a precious material for the new production of protein hydrolysates because their rich in many nutrients which show a good results of anti-inflammation activity and antioxidant activity. Prior to this study have little education about anti-inflammatory activity and antioxidant activity in family Mollusca. In this study, protein hydrolysate extract from spotted Babylon by using 3 enzyme and then survey effect of antioxidant activity and anti-inflammatory activity such as DPPH radical scavenging assay, ABTS radical scavenging assay, nitric oxide radical scavenging, Superoxide radical scavenging activity assay, glutathione peroxidase activity assay and others assay.

This study, therefore, proposed that spotted babylon might be the main source of protein hydrolysate. The objective of this study was to impose antioxidant scavenging activity and anti-inflammation of protein hydrolysate obtained from peptide of spotted babylon. So, spotted babylon hydrolysates are new peptides

which have a significant bioactive and interesting. Antioxidant activity and anti-inflammatory activity containing peptides from spotted babylon could be further developed for commercial products. Then, development is a good dosage form and low side effects by using compensates anti-inflammatory medicine which effects to digestive and expensive.

CHAPTER II

LITERATURE REVIEWS

2.1 Free radicals

Free radicals are atoms or molecule with an unpaired electrons and can be interacts with other molecules. Once formed these highly reactive radicals can start a chain reaction. Free radicals can be produced from endogenous or exogenous sources. Endogenous sources generating free radicals from normal cell metabolisms occur during aerobic respiration in the mitochondria. Exogenous source from air pollution, cigarette smoke, Ozone (O_3), alcohol, industrial solvents, cooking (smoked meat) medication and radiation(Pham-Huy, He et al. 2008). Free radicals include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), hydrogen peroxide (H_2O_2), peroxyl ($ROO^{\bullet}$) and hypochlorous acid (HOCl) (Lü, Lin et al. 2010).These free radicals may oxidize nucleic acids, proteins, lipids or DNA For example, hydroxyl radical and peroxynitrite can damage cell membranes and lipoproteins by a process called lipid peroxidation, which are cytotoxic and mutagenic. Lipid peroxidation occurs by a radical chain reaction. Proteins may also be damaged by free radical, leading to structural changes and loss of enzyme activity. Oxidative damage to DNA leads to the formation of different oxidative DNA lesions which can cause mutations(Pham-Huy, He et al. 2008). So, free radicals appears to be a major role to many diseases such as cancer, autoimmune disorders, aging, cardiovascular diabetes mellitus, pathogenesis of inflammatory and neurodegenerative diseases (Valko, Leibfritz et al. 2007).

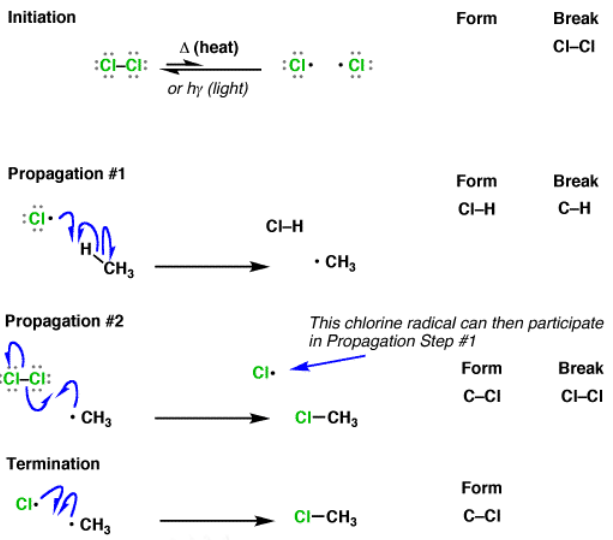


Figure 1 Free radicals chain reactions

The mechanism of free radicals chain reactions is chemical reaction. Radical chain reactions have three: initiation, propagation, and termination shown in Figure 1. The initiation phase describes initially creates a radical species. It is a hemolytic cleavage event and takes place very rarely due to the high energy barriers involved by the application of light, heat, UV radiation or a metal-containing catalyst is necessary to overcome the energy barrier. The propagation phase describes create the chain of chain reactions. Once a reactive free radical is generated, it can react with stable molecules to form new free radicals. These new free radicals go on to generate more free radicals, and start over again. Chain termination occurs when two free radical species react with each other to form a stable, non-radical adduct(Gordon 1990).

2.2 Antioxidant activity

An antioxidant is a molecule that inhibits the oxidation of free radicals. Antioxidants are molecules that can neutralize free radicals by accepting or donating electron(s) to eliminate the unpaired condition of the radical. The human body systems have antioxidants including enzyme and non-enzymatic compounds. The function of antioxidant is varied mechanisms of each compound including acting as radical scavenging, hydrogen donor, reducing agent, enzyme inhibitor and metal

chelating (Lobo, Patil et al. 2010). For example, non-enzymatic antioxidants have aromatic ring structures and are able to delocalize the unpaired electron such as vitamin C, vitamin E, B-carotene and uric acid. Enzymes antioxidant are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (Lü, Lin et al. 2010). These produced in the body provide an important defence against free radicals. Moreover**,** synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are widely used in the food industry. However, synthetic antioxidants is strictly regulated because of effects in some animals at high levels (Shahidi and Zhong 2010). Therefore, natural antioxidants from various natural sources as alternatives to synthetic as protein hydrolysate from plant and animal.

2.2.1 Non-enzymatic antioxidant

2.2.1.1 Vitamin C (Ascorbic acid)

Vitamin C is a water-soluble vitamin and can be found in a number of foods, such as fruits and vegetables. Vitamin C is not an enzyme, but classified as electron donor. Ascorbic acid is a reducing agent and can reduce by neutralize free radical such as hydrogen peroxide (Shigeoka, Ishikawa et al. 2002). Ascorbic acid play an important protective role in the body against the damaging effects of free radicals through its ability as an antioxidant to scavenge free radicals and to regenerate vitamin E (Bendich, Machlin et al. 1986).

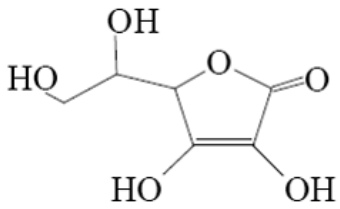


Figure 2 Vitamin C (Ascorbic acid)

2.2.1.2 Vitamin E (tocopherol)

Vitamin E is fat-soluble vitamin and can be found in wheat germ oil, sunflower, and safflower oils. It protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction. This reaction

produces oxidized **α**-tocopheroxyl radicals that can be recycled back to the active reduced form (Traber and Atkinson 2007).

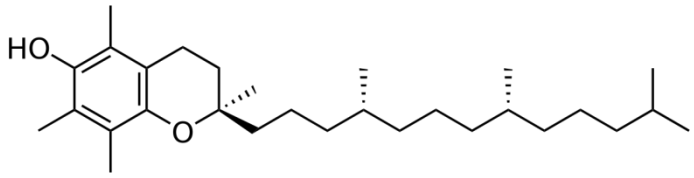


Figure 3 Vitamin E (tocopherol)

2.2.2 Enzyme antioxidant

2.2.2.1 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is an enzymes antioxidant that catalyze the breakdown of the superoxide anion (O_2^-) into oxygen and hydrogen peroxide (H_2O_2)**.** SOD is essential enzyme present in almost all aerobic cells and in extracellular fluids which is located in every part of living systems. There are three major families of superoxide dismutase, depending on the metal cofactor: Cu/Zn (which binds both copper and zinc) (Zelko, Mariani et al. 2002)**.**

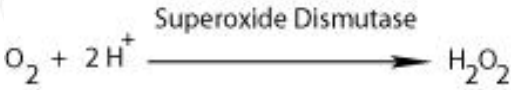


Figure 4 Superoxide dismutase(SOD)

2.2.2.2 Catalase (CAT)

Catalase (CAT) is the ferroprotoporphyrin (heme) containing enzyme, found in nearly all living organisms. This enzyme functions to catalyze the decomposition of hydrogen peroxide into less reactive water and oxygen (Chelikani, Fita et al. 2004).

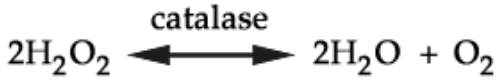


Figure 5 Catalase (CAT)

2.2.2.3 Glutathione peroxidase (GPX)

Glutathione peroxidase is an enzyme containing four selenium-cofactors that catalyze the breakdown of hydrogen peroxide(H_2O_2) and organic hydroperoxides

(ROOH) to water, using the tripeptide glutathione as a hydrogen donor(Brigelius-Flohé 1999).

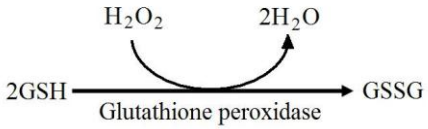


Figure 6 Glutathione peroxidase (GPX)

2.3 Anti-inflammation activity

2.3.1 Inflammation

The inflammation signs are pain, heat, redness, swelling, and loss of function. Inflammation can be classified into 2 cases including acute and chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. Chronic inflammation or prolonged inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process (Calder, Albers et al. 2009). During inflammation, macrophages cells release chemical mediators such as nitric oxide and prostaglandins E2. These substances built from leukocytes by enzymes Cycloxigenase-2 (COX-2) and Inducible Nitric oxide synthase (iNOS). Nitric oxide and prostaglandins E2 Will be induced to create up a large number of cytokine and endotoxin. However, inflammations have both beneficial effects and harmful effects as shown in Table 1. And an important role in the disease of inflammation such as rheumatoid arthritis, atherosclerosis and asthma. (Hanada and Yoshimura 2002) Therefore, determination of anti-inflammatory properties has been proposed as a good way for preventing it.

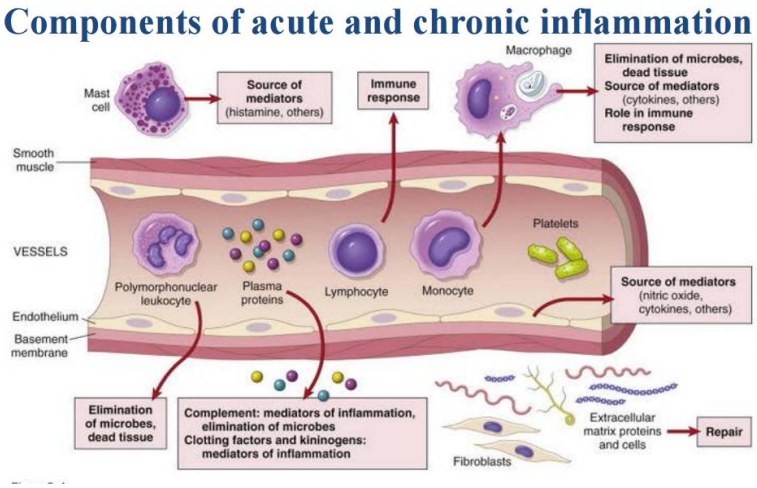


Figure 7 Acute and chronic inflammation

Table 1 Beneficial effects and harmful effects of inflammation

Beneficial effects of inflammation	Harmful effects of inflammation
Dilution of toxins	Persistent cytokine release
Entry of antibodies	Destruction of normal tissues
Fibrin formation	Swelling
Delivery of nutrients	Inappropriate inflammatory response
Oxygen Stimulation of immune response	

2.3.2 Biological of nitric oxide

Nitric oxide is a chemical compound with chemical formula of NO which has an effective molecule in many biological systems such as immunological, neuronal, cardiovascular, tissues, blood circulatory system. NO is one of the inflammatory mediators that have been implicated in a variety of pathophysiological conditions including inflammation, 4 carcinogenesis and atherosclerosis. NO acts as a host defense by damaging pathogenic DNA, and as a regulatory molecule with homeostatic activities (Kou and Schroder, 1995). Moreover, the potentially lethal substances released from microglia, overproduction of NO has been reported to cause oxidative damages and cell death (Minghetti and Levi, 1998). In animal cells,

NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). Three NOS isoforms have been identified neuronal NOS (nNOS), inducible NOS in macrophages (iNOS), and endothelial NOS (eNOS) (Table 2) (Nathan and Xie, 1994). The nNOS and the eNOS are referred to as constitutive NOSs, whereas iNOS gene expression is induced in macrophages and many other cell types in response to inflammatory agents and cytokines (Mayer and Hemmens, 1997).

Table 2 Classification of mammalian nitric oxide synthase

Isoform	Typical localization	Cellular localization	Monomer molecular mass (kDa)
nNOS	Neuron	Soluble and particulate	-160
eNOS	Endothelial cells	Particulate	-135
iNOS	Macrophages	Soluble	-130

Inducible NOS (iNOS) is induced in response to various pro-inflammatory cytokines, including interferon-**γ** (IFN-**γ**), tumor necrosis factor-**α** (TNF-**α**), and interleukin-6 (IL-6), and mediates several inflammatory responses. NO synthesized by iNOS has also been considered as an important mediator of carcinogenesis. Overproduction of NO has been reported to cause oxidative damage cell death. Inhibition of NO overproduction has been proved to increase cell survival in several models. For example, NO has been correlated 8 with the inflammatory process, in which multiple cytotoxic effects are related to the ability to increase vascular permeability and edema. This involves changes in local blood flow and increases in pro-inflammatory prostaglandins (Lantz et al., 2005). The macrophages can be activated by lipopolysaccharide (LPS) and interferongamma (IFN-**γ**). LPS is one of the major constituents of the outer membrane of Gramnegative bacteria, and the immune system is constantly exposed to low levels of LPS through infections. LPS recognition and signal transmission are the key events aimed at eliminating an invading pathogen. The LPS-induced activation of macrophages results in the production of bioactive lipids, reactive oxygen species, and in particular,

inflammatory cytokines to fight and clear the bacterial infection. However, LPS mediates both the beneficial and deleterious reaction to the host. The excessive and uncontrolled production of inflammatory mediators triggered by LPS is harmful, and can lead to potentially lethal systemic disorders such as septic shock (Cho et al., 2008). The antigen can activate macrophages to release some inflammatory mediators such as NO, TNF-**α**, PGE2 and others. Therefore, the inhibition of NO, PGE2 and TNF-**α** production is an important therapeutic consideration in the development of anti-inflammatory agents.

2.3.3 Nitric oxide activity

Measurement of NO generation can be performed by different experimental setups. The rate of conversion of 3 H- or 14C- labeled L-arginine into the respective labeled L-citrulline and subsequent spectrophotometry is a standard procedure for indirect confirmation of NO production. L-citrulline can be readily detected using ionexchange procedures to separate substrate and product (Chan et al., 1997). In addition, there are many spectrophotometric assays available for the determination of NO metabolites. In oxygenated solution, NO reacts with O_2 to form nitrite and nitrate which can be measured using the Griess reaction (Kelm et al., 1997). The Griess Reagent System is based on the chemical reaction shown in Figure 8, which uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. This system detects NO_2 in a variety of biological and experimental liquid matrices such as plasma, serum, urine and tissue culture medium (Griess, 1879).

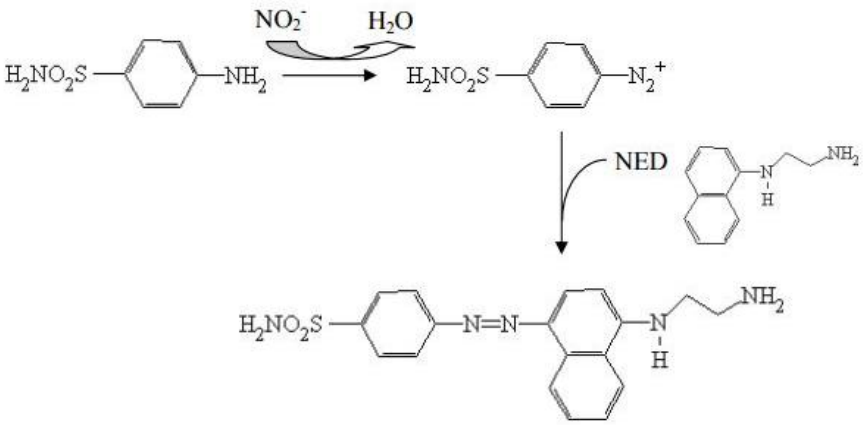


Figure 8 Chemical reaction of NO_2 using the Griess Reagent System

2.3.4 Cytotoxic activity

The predictive value of in vitro cytotoxicity tests is based on the idea of "basal" cytotoxicity – that toxic chemicals affect basic functions of cells which are common to all cells, and that the toxicity can be measured by assessing cellular damage. The development of in vitro cytotoxicity assays has been driven by the need to rapidly evaluate the potential toxicity of large numbers of compounds, to limit animal experimentation whenever possible, and to carry out tests with small quantities of compound. Evidence for the utility of in vitro cytotoxicity tests (Barile et al., 1994; Davila et al., 1990; Todd et al., 1999). The general principles for the detection of cell growth or cell kill via the MTT cytotoxicity assay (Figure 9). This method has been proven to be user friendly, rapid and highly sensitive (Promega, 2005; Bakand et al., 2006; Potera, 2007). MTT (3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in living cells. A solubilization solution (usually dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed (Mosmann, 1983).

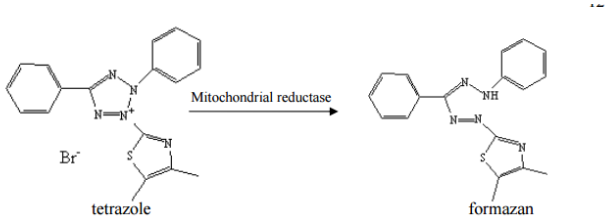


Figure 9 MTT assay

2.3.5 Anti-inflammation protein

The anti-inflammatory activity of several protein hydrolysate and isolated compounds has already been reported. Protein hydrolysate of tuna cooking juice showed the peptide fractions with the molecular weight ranging from 204 to 1672.9 Da possessed the highest anti-inflammatory activity. The amino acid sequences of the two anti-inflammatory peptides were Pro-Arg-Arg-Thr-Arg-Met-Met-Asn-Gly-Gly-Arg (1543.8 Da) and Met-Gly-Pro-Ala-Met-Met-Arg-Thr-Met-Pro-Gly (1211.5 Da) (Cheng, Wang et al. 2015). The peptic hydrolysates from salmon byproduct protein showed anti-inflammatory activity by inhibiting nitric oxide production and proinflammatory cytokines including tumor necrosis factor-α, interleukin-6 and -1β in RAW264.7 macrophage cells. The peptides contained amino acids such as Tyr, Phe, Pro, Ala, His, and Leu (Ahn, Je et al. 2012). In 2013, Wang and co-workers were reported that blue mussel protein hydrolysed by neutrase exhibited the highest DPPH radical scavenging activity. BNH-P7, the fraction departed from HPLC, exhibited good scavenging activity on DPPH radical, hydroxyl radical, and superoxide anion radical with IC50 of 2.62, 0.228, and 0.072 mg/ml, respectively. In 2015, Ahn and co-workers were reported the anti-inflammatory peptide from salmon hydrolysated by pepsin. The purified peptide inhibited the production of NO by 63.80% and PGE2 by 45.33%.

2.4 Preparation Protein hydrolysate

Protein hydrolysate from plan and animal containing peptide or amino acid 3-20 amino acids. The protein hydrolysate can be produced by acid-base extraction or enzymatic hydrolysis. In this thesis, enzymatic hydrolysis was chosen to selectively hydrolyze protein because enzymatic hydrolysis is non-toxic chemicals in the

products. The advantages and disadvantages of enzymatic hydrolysis as shown in Table 3. Protein hydrolysis by enzyme including protease such as protease from animal (trypsin, pepsin and pancreatin), protease from plant (papain and protease), protease from microorganism (alcalase, , flavourzyme and neutrase) under mind condition of pH (6-8) and temperature (40-60 °C) (Table 4) (Ahn, Cho et al. 2015).

Table 3 The advantages and disadvantages of enzymatic hydrolysis

Advantages	Disadvantages
1. Not-consumed during reaction	1. Instability
2. High conversion yield	2. High cost
3. Nontoxic and biodegradable	
4. High specificity	
5. Utilization in soft conditions	
6. Large-scale production	

Table 4 Proteolytic enzymes, characteristics and optimum conditions

Enzyme	Source of origin	Condition	
		pH	Temp (°C)
Alcalase	*Bacillus licheniformis*	7.0	50
Flavourzyme	*Bacillus stearothermophilus*	7.0	50
Neutrase	*Bacillus amyloliquefaciens*	7.0	50
Pepsin	Porcine gastric mucosa	2.0	37
Trypsin	Bovine, porcine or human pancreas	8.0	37
Papain	*Carica papaya* (papaya latex)	6.0	37

2.5 Bioactive activity from marine source

Although, synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are strictly regulated because of effects in toxicity

(Shahidi and Zhong 2010). Therefore, natural antioxidants from various natural sources as alternatives to synthetic such as protein hydrolysate from plant and animal. The marine source, which makes up more than 70% of the earth's surface, represents a vast with diverse biological activities have been identified in mollusk, algae, fish and shellfish, which represents a good candidate raw material for protein hydrolysate peptide.

In 2012, Bougatef and co-workers were studied sardine fish '*Sardinella aurita*'. They hydrolyzed the crude protein by NH1 protease enzyme for 3 hours. The antioxidant activity is about 87 percentages.

In 2013, Wang and co-workers were studied blue mussel shell '*Mytilus edulis*'. They hydrolyzed the crude protein by neutrase enzyme for 3 hours. The antioxidant activity is about 28 percentages.

In 2014, Jiang and co-workers were studied round scad fish '*Decapterus maruadsi*'. They hydrolyzed the crude protein by alcalase enzyme for 5 hours. The antioxidant activity is about 39 percentages.

There are many proteins hydrolysate from marine sources including plants and animals which represent antioxidant activity such as *Ecklonia cava* (microalgae) (Heo, Jeon et al. 2003), Porphyra tenera(macroalgae) (Lee, You et al. 2005). Penaeus japonicus (prawn) (Suetsuna 2000). Mytilus edulis(mussel) (Rajapakse, Mendis et al. 2005) and Dosidicus eschrichitii (squin) (Lin and Li 2006). Morover, proteins hydrolysate from marine sources shown bioactive activity including antihypertensive, ACE inhibitory, anti-inflammation, antimicrobial, antityrosinase and antitumor. Such as Harengula zunasi(sardine) (Shang-gui, Zhi-ying et al. 2004), Tachypleus tridentatus (Crab) (Masuda, Nakashima et al. 1992), Parasilurus asotus(cat fish) (Park, Park et al. 1998), Scomber austriasicus(maceral) (Masaaki and Hiroyuki 2007), Dolabella auricularia(sea hare) (Madden, Tran et al. 2000) and Pomatomus saltatrix (Trout) (Bauchart, Chambon et al. 2007).

Table 5 Some bioactive activity of protein hydrolysates from marine source

Marine source	Bioactive activity	Peptide sequence	References
Porphyra yezoensis	Antihypertensive	AKYSY	Saito and Hagino 2005
Gadus microcephalus (Cod)	Antioxidant	TGGGNV	Ngo, Ryu et al. 2011
Pinctadafucata martencii (Oyster)	ACE inhibitory	PY	Katano, Oki et al. 2003
Mytilus edulis (Mussel)	Antihypertensive	EVMAGNLYPG	Je, Park et al. 2005
Dosidicus giga (Squin)	antioxidant	NADFGLNGLE GLA	Rajapakse, Mendis et al. 2005
Meretrix lusoria (Clam)	ACE inhibitory	VRK	Tsai, Chen et al. 2008
Homarus gammarus (Lobster)	Antimicrobial	-	Hauton, Brockton et al. 2006
Bryopsin sp. (Trout)	Antitumor	-	Ciruelos, Trigo et al. 2002

2.6 Protein hydrolysate from Mollusca

Mollusca is one of the most diverse groups of marine animals with at least 50,000 living species includes snails, octopuses, squid, clams, scallops, oysters, and chitons. Some of Mollusca have report about bioactivity activity such as antioxidant activity, anticoagulant, antihypertensive and antimicrobial as shown in Table 6. Antioxidant activity of protein hydrolysates relies on amino acid composition of sequence and molecular weight of a peptide. Peptides containing hydrophobic amino acids, such as Pro, Leu, Ala, Trp and Phe, are possess high antioxidant activity (Mendis, Rajapakse et al. 2005). Peptides containing aromatic amino acid such as Trp, Tyr and Met also show the highest antioxidant activity, followed by Cys, His and Phe (Dávalos, Miguel et al. 2004).

Table 6 Bioactive activity of protein hydrolysate of Mollusca

Source	Bioactive activity	Amino acid sequence	Referent
Oyster	Antioxidant	LKQELEDLLEKQE	Qian, Jung et al. 2008
Mytilus edulis (Blue mussel)	Antioxidant	HFGDPFH	Rajapakse, Mendis et al. 2005
Mytilus edulis	Anticoagulant	EADIDGDGQVAMMTSK	Jung and Kim 2009
Hard clam	Antihypertensive	YN	Tsai, Chen et al. 2008
Crassostrea gigas (Oyster)	Inhibit HIV-1 protease	LLEYSL	Lee and Maruyama 1998
Crassostrea gigas (Oyster)	Antimicrobials	LLEYSI LLEYSl	Liu, Dong et al. 2008
Mytilus galloprovincialis.	Antimicrobial	-	Mitta, Hubert et al. 1999

2.7 Spotted babylon (*Babylonia areolata*)

Figure 10 Spotted babylon (*Babylonia areolata*). (Source; http://www.ku.ac.th/e-magazine/jan51/agri/Babylonia.htm)

Babylonia areolata or Spotted babylon, belonging to the family Buccinidae, genus Babylonia, is an important gastropod consumption in Thailand. Then, spotted

babylon's shell has heavy and oval which has white color and brown stripes on shell. They have highlights organ such as 2 eyes, 2 tentacle, shell, big foot uses moment like snail. Afterwards these shells eat feed by using organ same as white tubing releases fluid which digests and suck feed. After that, it moves in the sandy. The feed of these ones are carrion from the sea, and then that grows by about 40-100 mm. Habitat of spotted Babylon is according to the sandy sea floor or Sandy mud from the depths 2 to 20 m which founds in Gulf of thailand. Spotted babylon has a high nutrition which contains 18.78% of protein, 17 amino acids (glutamic acid arginine and alanine up to 80.18% of the total free amino acid contents), fat 2.86%, carbohydrate 5.18%, ash 5.27%, and moisture 67.91% (Chaitanawisuti and Kritsanapuntu 1999). meat and intestines of spotted babylon includes 17 amino acids. Nowadays, spotted Babylon is a kind of economic animals and eats together in both domestic and foreign. Therefore, the promotion of aquaculture is a career.

Table 7 Comparison of nutritional values of spotted babylon with other economically important marine organisms

Organism	Composition of nutritional value (%)				
	Protein	Fat	Ash	Carbohydrate	Moisture
Natural spotted babylon	18.03	2.39	2.38	3.33	73.87
Cultivated spotted babylon	20.01	6.33	2.04	0.79	70.83
Mussel	11.90	2.24	1.59	3.69	80.58
Oyster	9.45	2.30	1.23	4.95	82.06
Abalones	17.10	0.76	1.57	6.01	74.56
Tiger prawn	20.31	1.73	1.20	0.90	75.86

Taken from (Prakot, Chaitanawisuti et al. 2014).

Table 8 Amino acid content (mg/100mg) in small and big size of spotted Babylon

Amino acid	Big size of spotted babylon	Small size of spotted babylon
Threonine	731.55	502.07
Isoleucine	1,071.44	767.10
Leucine	2,211.60	1,948.33
Lysine	2,792.39	2,714.73
Methionine	300.32	303.42
Cystine	35.54	18.55
Phenylalanine	1,171.00	1,851.35
Tyrosine	1,624.58	1,321.69
Valine	1,031.18	725.92
Arginine	229.81	131.89
Histidine	1,540.16	1,212.00
Alanine	1,149.63	788.54
Aspartic acid	1,006.29	477.89
Glutamic acid	1,133.58	1,433.07
Glycine	1,274.81	874.41
Proline	1,749.84	1,147.63
Serine	22.06	17.31

Translated from (Chaitanawisuti and Kritsanapuntu 1999).

Table 9 Amino acid composition of spotted babylon *Babylonia areolata* (mg/100mg).

Amino acid profile	Results (%)
Alanine	4.36
Arginine	5.85
Glycine	4.30
Aspartic Acid	7.09
Valine	3.03
Cystine	0.64
Glutamic Acid	11.70
Leucine	5.27
Isoleucine	2.36
Histidine	1.20
Threonine	3.15
Proline	3.12
Lysine	4.42
Methionine	1.65
Hydroxyproline	Not Detected
Serine	3.12
Phenylalanine	2.43
Hydroxylysine	Not Detected
Tyrosine	2.37
Tryptophan	0.40

Taken from; (Prakot, Chaitanawisuti et al. 2014)

CHAPTER III

MATERIALS AND METHODOLOGY

3.1 Peptide preparation

Spotted babylon (Sichang Marine Science Research, Chulalongkorn University in Cholburi province of Thailand) was asphyxiated with ice. Then, Spotted babylon removes shell by hammer. The muscle of spotted babylon was separated manually and washed with cold distilled water. After that the muscle of spotted babylon was pounded to homogenate (Kim, Kim et al. 2013). Crude protein was extracted from spotted babylon using 20 mM phosphate buffer (pH 7.2). Three proteases; alcalase, flavourzyme and neutrase, were used in the hydrolysis process. Five percentages of enzymes were mixed with crude protein and incubated at 50 °C for 4 h prior to inactivation of the protein by boiling at 90 °C for 20 min. Finally, all protein hydrolysis was silted by centrifugation. Afterward, protein hydrolysis was collected from supernatant and remove sediment. Spotted babylon hydrolysate (SBH) was kept in a cold storage at -20 °C until desired for using.

3.2 Purification and identification

3.2.1 Ultrafiltration

All protein hydrolysates were fractionated by ultrafiltration using the molecular weight cut-off membrane of 10 (Amersham Biosciences, Sweden). Protein hydrolysates were initially processed in a 10 kDa membrane to generate a retentate (MW > 10 kDa) and permeate (MW < 10 kDa). The permeate proteins (MW < 10 kDa) were collected for antioxidant activity assay and purification (Jiang, Tong et al. 2014).

3.2.2 Gel filtration chromatography

The fraction with the best antioxidant activities was further purified by injection into a Sephacryl S-200 gel filtration column (2.6 × 80 cm) equilibrated with ultrapure water, and the loaded proteins were eluted with ultrapure water at a flow rate of 1.0 ml/min (Ahn, Cho et al. 2015). Each fraction of the eluted solution was detected at 280 nm. Their antioxidant activities were examined. The fractions with the highest antioxidant activities was subjected to the next purification step.

3.2.3 Reversed-phase high-performance liquid chromatography (RP-HPLC)

The fraction with the highest antioxidant activities from the gel filtration chromatography was further purified by reversed phase high performance liquid chromatography on Shimpack C-18 column (250×46 mm), using a linear gradient of acetonitrile containing 0.05% trifluoroacetic acid (TFA) (Bougatef, Ali et al. 2010). The elution solution was detected at 280 nm and 3 purified peptides, namely F1, F2, and F3, were analyzed by mass spectrometry.

3.2.4 Mass spectrometry

Mass spectrometry is an analytical chemistry technique to identify compounds or peptides present in a sample by measuring the mass-to-charge ratio (m/z) and abundance of gas-phase ions. The objective of this study was to identify compounds in protein hydrolysate by LC-MS/MS Q-TOF data were analyze by de novo peptide sequencing.

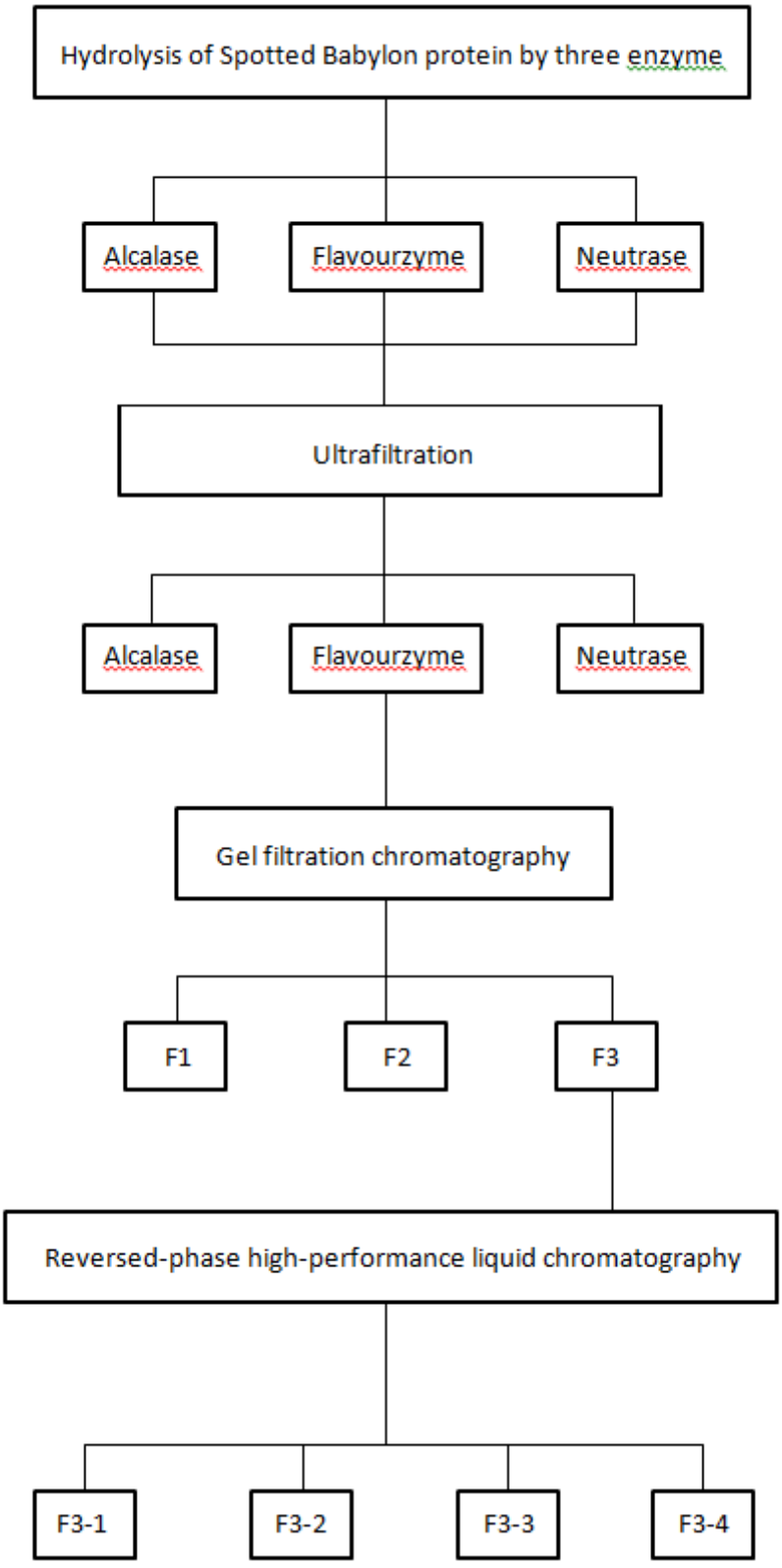


Figure 11 The entire process of this work.

3.3 Protein concentration assay

Protein contents of the sample were determined using the Bradford assay. Various concentrations of bovine serum albumin (BSA) were used as the standard for the constructing of calibration curve (Bradford 1976).

3.4 DPPH radical scavenging assay

The DPPH radical scavenging activity was performed according to the method of Mohsen and Ammar (Mohsen and Ammar 2009) with slight modifications. of Protein hydrolysate (40 µl) was added to 160 µl of DPPH solution (1M in 100% of ethanol). The mixtures were incubated in the dark at room temperature for 30 min, and reduction of DPPH radicals was measured at 517 nm using a microplate reader. L-ascorbic acid (Vitamin C) was used as a standard control .

3.5 ABTS radical scavenging assay

The ABTS radical scavenging activity was determined according to the method of Cai et al (Cai, Luo et al. 2004) with slight modifications. ABTS solution was prepared from 7 mM ABTS in potassium persulphate solution. ABTS solution was incubated in the dark at room temperature for 12 h. The ABTS solution (300 µl) was mixed with 10 µl of protein hydrolysate, and the mixtures were incubated at room temperature for 5 min. The reduction of ABTS radicals was measured at 734 nm using a microplate reader.

3.6 Nitric oxide radical scavenging

The nitric oxide radical scavenging was performed according to the method of Govindarajan et al (Govindarajan, Rastogi et al. 2003). Protein hydrolysate was mixed with Griess containing 10 mM sodium nitroprusside in 20 mM buffer solution (pH 7.2). The total volume was then adjusted to 40 µl, and the mixtures were incubated at room temperature for 150 min. of The mixtures (0.5 ml) were added to 100 µl of sulfanilamide solution (0.33% sulfanilamide in 20% acetic acid), and incubated for 5 min at 37 °C. 100 µl of N-(1-naphthyl) ethylene diamine dichloride (NED) was added and incubated at at room temperature for 30 min, then the absorbance at 540 nm was measured by a microplate reader.

3.7 Superoxide radical scavenging activity assay

The assay of superoxide dismutase was measured the method of 19160 SOD determination kit by sigma-aldrich. In this method, 20 µl of each protein hydrolysate were mixed with 200 µl WST working solution (WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2Htetrazolium, monosodium salt). Add 20 µl Enzyme working solution and mix thoroughly. The mixture incubated at 37°C for 20 min. The absorbance was measured at 450 nm.

3.8 Catalase activity assay

Catalase activity was assayed by the method of Catalase assay kit by sigma-aldrich. Catalase is an antioxidant enzyme that is present in living organisms. Catalase activity can be determined in the measurement of the hydrogen peroxide substrate remaining after the action of catalase (Catalase change hydrogen peroxide (H_2O_2) to water and oxygen) In this method, add 20 µl of protein hydrolysate to quartz plate mixing with 80 µl of 1x Assay Buffer. The reaction was started by the addition of 100 ml of UV Assay Substrate Solution (20 mM H2O2). Follow the decrease of A240 for 30 sec with the kinetic program, the following parameters: Initial delay = 3 seconds, interval = 5 seconds, readings = 7

The activity was calculated as follow:

$$\text{Unites/ml} = \frac{[\Delta A/\min(Blank) - \Delta A/\min(Sample)] \times d \times 1}{V \times 0.0436}$$

d = dilution of original sample, V= sample volume (x µl = 0.00x ml), 0.0436 = ε^{mM} for hydrogen peroxide , 1= reaction volume in ml

3.9 Glutathione peroxidase activity assay

Glutathione peroxidase activity was assayed by the method of Glutathione peroxidase cellular activity assay kit sigma-aldrich. The assay kit uses an indirect determination method base on GPx converts reduced glutathione (GSH) to oxidized glutathione (GSSG). In this method, add 180 µl into quartz plate. Then, add 10 µl of NADPH Assay Reagent and 8 µl of protein hydrolysate and mix by inversion. The reaction was started by the addition of 2 µl of 30 mM tert-Butyl Hydroperoxide

Solution. And mix by inversion. Follow the decrease in absorbance at 340 nm with the kinetic program, the following parameters:

Initial delay = 15 seconds, Interval = 10 seconds, Readings = 6

The activity was calculated as follow:

$$\text{Unites/ml} = \frac{(\Delta A_{340}/min_{(blank)} - A_{340}/min_{(sample)}) \times DF}{6.22 \times V}$$

DF = dilution factor of original sample, V= sample volume in ml, 6.22 = ε^{mM} for NADPH

3.10 The percentage inhibition

The percentage inhibition of antioxidant activity was calculated as follows:

$$\left[\frac{(Abs\ \text{control} - Abs\ \text{blank}) - (Abs\ sample - Abs\ \text{background})}{(Abs\ \text{control} - Abs\ \text{blank})}\right] \text{x } 100$$

where Abs control is the absorbance of control (no sample), Abs sample is the absorbance of sample, Abs background is the color absorbance of the sample, and Abs blank is the absorbance of deionized water. The IC50 was calculated using GraphPad Prism version 6. All tests were performed in triplicate. The values are expressed as mean ± standard deviation of the triplicate data.

3.11 RAW 264.7 cell culture and pretreatment

The RAW 264.7 cell line cultured in DMEM medium added with 10% (v/v) fetal bovine serum (FBS) (Chantaranothai, Palaga et al. 2013). RAW 264.7 cells were incubated at 37°C in the presence of 5% CO2 in Forma™ 310 Direct Heat CO_2 Incubators. Cell culture uses times about 3-4 day. After that, the medium was pulled and the cells were rinsed out with 0.5 ml of trypsin at 30-40 minutes. Trypsin was removed and added new DMEM medium. Then, all cells in DMEM medium were collected in tube for determination of nitric oxide and MTT assay. The viable cell numbers were calculated following equation.

Total cell count (cells/mL)

= the number of cell counted in 4 large squares x 10 x 10^4

After that, total cell count mix new DMEM medium in sterilized bottle. Then, total cell from bottle were suck to 180 µl/well of 96-well plates which were incubated overnight at 37°C in 5% CO_2. Therefrom preparation of sample and LPS (lipopolysaccharide), sample was mixed DMEM medium at the ratio 1:2 to 1:1,000 (v/v) and 1 µg/ml of LPS was prepared from 500 µg/ml of stock LPS. Next day, DMEM medium was removed from 96-well plates and then 96-well plates was added at 190 µl/well of sample and 10 µl/well of LPS. 96-well plates were incubated overnight at 37°C in 5% CO_2.

3.12 Inhibition of nitric oxide (NO) production From RAW 264.7 Cells

RAW264.7 macrophage cells were incubated incubated overnight at 37°C in 5% CO_2. with different concentrations of sample in the absence or presence of 10 µl of LPS. After that, 100 µl of culture medium was collected and used to NO assay. Then, 100 µl of culture medium was mixed with 100 µL of Griess reagent (0.33% sulfanilamide in 1% acetic acid and 0.1% N-(1-naphthyl) ethylene diamine dichloride), and further incubated for 15 min, then the absorbance at 540 nm was measured by a microplate reader. The Sodium nitrite dissolved in DMEM was. using a standard curve of NO concentration in the media of sample healed cells.

3.13 MTT assay for measuring cell proliferation

The MTT assay (Yang, Kang et al. 2014) was assessed cytotoxicity of sample from RAW264.7 macrophage cells were grown in 96-well plates at a density of 1.6×10^4 cells/well. After overnight, RAW264.7 macrophage cells were treated with control medium (medium containing only different concentration sample) and medium containing different concentration sample and LPS which were collected overnight or 24 h at 37°C in 5% CO_2. After that, The MTT solution (1 mg/mL) was added 10 µl/well in 96-well plates cell culture, and incubated for 4 h. Finally, dimethyl sulfoxide (DMSO) was added 100 µl/well in 96-well plates due to DMSO solubilizes the formed formazan crystals. The volume of formazan crystals was determined by measuring the absorbance at 540 nm.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined by mixing of the protein sample and DPPH solution, and measured the reduction of DPPH radicals at 517 nm. In DPPH scavenging activity, the violet compound of DPPH radical turns yellow when neutralized by antioxidant. The DPPH radicals scavenged and the absorbance was reduced. The reduction in absorbance is therefore used to determine radical scavenging activity. The results of DPPH radical scavenging activity from spotted babylon protein were shown as changes of IC_{50} values (Tables 10 and 11). All protein hydrolysate were found to have the efficacy to scavenge the DPPH radical and showed no significant difference ($p > 0.05$). The protein hydrolysates that were passed to ultrafiltration membrane (MW < 10 kDa) flavozyme hydrolysate was found to have the highest scavenging activity (0.75 ± 0.04 μg/ml) which have scavenging activity more than protein hydrolysate from *Decapterus maraudsi* (Jiang et al., 2014) and *Mytilus edulis* (Wang et al., 2013), while alcalase hydrolysate was found to have the lowest DPPH radical-scavenging activity (1.27 ± 0.24 μg/ml).

4.2 ABTS radical scavenging activity

ABTS radical can be determined by monitoring the absorbance at 734 nm. Antioxidants suppress this reaction by electron donation radical scavenging and inhibit ABTS radical. The results of ABTS radical scavenging activity expressed as IC_{50} values are shown in the Table 10. All protein hydrolysate had the efficacy to scavenge. The highest ABTS scavenging activities were obtained from alcalase and flavozyme hydrolysate (5.28 ± 0.21 and 3.78 ± 0.08 μg/ml, respectively), whereas the lowest ABTS radical-scavenging activity was obtained from neutrase hydrolysate (8.00 ± 0.28 μg/ml). Table 11 shows that the IC_{50} values of protein hydrolysate whose molecular mass was less than 10 kDa and with the highest IC_{50} values of ABTS radical scavenging activity were alcalase and flavozyme hydrolysate (2.47 ± 0.11 and 2.90 ± 0.12 μg/ml, respectively), whereas neutrase hydrolysate had the lowest ABTS radical scavenging activity (1.27 ± 0.24 μg/ml). The results of ABTS scavenging activity in

many researchers found EC50 of LPF (1.014 mg/ml), LLPF (1.031 mg/ml), from the protein hydrolysate of scalloped hammerhead, (Luo et al., 2013) and corn gluten meal (Zhuang et al.,2013), respectively.

4.3 Nitric oxide scavenging activity

Nitric oxide was formed from sodium nitropusside. All protein hydrolysates exhibited antioxidant activity. Nitric oxide was decreased and could be measured by monitoring the absorbance at 540 nm. The nitric oxide scavenging activity was shown as IC_{50} values in Tables 10 and 11. The highest nitric oxide scavenging activity of crude protein was from flavourzyme hydrolysate and neutrase hydrolysate (0.005 ± 0.17 and 0.007 ± 0.19 µg/ml, respectively), but alcalase hydrolysate exhibits lowest nitric oxide scavenging activity which is 0.056 ± 0.068 µg/ml. The protein hydrolysate (MW<10 kDa) with the highest nitric oxide scavenging activity were those from flavozyme and neutrase hydrolysate (0.0017 ± 0.00037 and 0.002±0.0004 µg/ml, respectively). Then, alcalase hydrolysate shows lowest nitric oxide scavenging activity look like control. Eun-Kyung Kim et al. (2013) report the effect of flavouzyme hydrolysate from *Mytilus coruscus* on the viability of RAW264.7 cells was examined by MTTassay and NO-inhibitory effect assay. The cell viability was not reduced by Flavouzyme hydrolytsate up to 1.0 mg/ml and NO reduced.

Table 10 IC_{50} values of DPPH, ABTS, nitric oxide activity from spotted babylon protein hydrolysate. Antioxidant activity assay by DPPH, ABTS, nitric oxide activity. Values are mean ± standard deviation (SD) (n = 3).

Enzyme	IC_{50}(μg/ml)		
	DPPH*	ABTS**	NO***
Control	6.56 ± 2.78[a]	12.88 ± 1.09[c]	0.016 ± 0.09[ab]
Alcalase	2.54 ± 0.34[a]	5.28 ± 0.21[a]	0.056 ± 0.068[b]
Flavourzyme	1.87 ± 0.98[a]	3.78 ± 0.08[a]	0.005 ± 0.17[a]
Neutrase	2.41 ± 2.66[a]	8.00 ± 0.28[b]	0.007 ± 0.19[a]
Ascorbic acid (positive control)	38.21	125.7	260.2

Control = spotted babylon protein wasn't digested from enzyme.

*DPPH scavenging effects of proteins were digested from 3 enzymes.

**ABTH scavenging effects of proteins were digested from 3 enzymes.

***NO scavenging effects of proteins were digested from 3 enzymes.

Table 11 Ultrafiltration separation (MW < 10 kDa) of protein hydrolysate from spotted babylon prepared by alcalase, flavourzyme and neutrase. Antioxidant activity assay was determined by DPPH, ABTS, nitric oxide activity. Values are the mean ± standard deviation (SD) (n = 3).

Enzyme	MW (kDa)	IC_{50}(μg/ml)		
		DPPH*	ABTS**	NO***
Control	< 10 kDa	4.10 ± 0.05[c]	15.07 ± 0.62[c]	0.034 ± 0.0019[b]
Alcalase	< 10 kDa	1.27 ± 0.24[b]	2.47 ± 0.11[a]	0.04 ± 0.007[b]
Flavourzyme	< 10 kDa	0.75 ± 0.04[a]	2.90 ± 0.12[a]	0.0017 ± 0.00037[a]
Neutrase	< 10 kDa	1.12 ± 0.09[ab]	4.13 ± 0.33[b]	0.002 ± 0.0004[a]
Ascorbic acid (positive control)		38.21	125.7	260.2

Control = spotted babylon protein wasn't digested from enzyme.

*DPPH scavenging effects of proteins were digested from 3 enzymes.

**ABTH scavenging effects of proteins were digested from 3 enzymes.

***NO scavenging effects of proteins were digested from 3 enzymes.

4.4 Superoxide dismutase radical scavenging activity

Another important free radical is superoxide radical which encourage oxidative reactions in body because superoxide radical scavenging activity stimulates the dismutation of the superoxide anion (O_2.-), and then particularly important as the product of the one-electron reduction of O_2. Table 12 shows % inhibition superoxide radical. Flavourzyme hydrolysate of crude protein obtains highest % inhibition of superoxide radical scavenging activity that is 73.7±0.92. The second is 64.66±0.21% inhibition of neutrase hydrolysate. Then percent inhibition of alcalase, and control are 59.45±2.86 and 53.62±2.79, respectively. All MW < 10 kDa of protein hydrolysate have value about 60-30 %inhibition.

Table 12 Crude protein and MW < 10 kDa of protein hydrolysate from spotted babylon prepared by alcalase, flavourzyme and neutrase. was measured percent inhibition of superoxide radical

Enzyme	SOD (%inhibition)	
	Crude protein	<10kDa
Alcalase	59.45±2.86[a]	59.4.±4.45[a]
Flavourzyme	73.7±0.92[b]	58.72±4.61[a]
Neutrase	64.67±4.27[a]	53.36±4.73[b]
Control	53.62±2.79[c]	34.81.±7.73[c]

4.5 Catalase activity

Catalase is enzyme found in nearly all living organisms revealed to oxygen, and then. It stimulates the decomposition of hydrogen peroxide to oxygen and water. Catalase is also an important antioxidant activity in body. This experiment shows results of catalase activity in Table 13. The highest catalase activity is

49.40±4.59 unit/ml of alcalase hydrolysate. Then, the second is 46.56±2.23 unit/ml of flavourzyme hydrolysate. Other samples have less values than 30 unit/ml.

Table 13 Crude protein and MW < 10 kDa of protein hydrolysate from spotted babylon prepared by alcalase, flavourzyme and neutrase. was measured unit/ml of catalase activity

Enzyme	Catalase (unit/ml)	
	Crude protein	<10kDa
Alcalase	23.43±2.20[a]	49.40±4.59[a]
Flavourzyme	17.51±1.81[b]	46.56±2.23[a]
Neutrase	17.11±0.94[b]	29±3.13[b]
Control	8.88±1.31[c]	12±0.81[c]

4.6. Glutathione peroxidase activity

Glutathione peroxidase is peroxidase activity whose important biological role is to protect the organism from oxidative damage. Then, The function of glutathione peroxidase reduces lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. As shown in Table 14, the highest value is 6.2625±0.93 unit/ml of neutrase hydrolysate (MW < 10 kDa). The second is 5.8625±2.31 unit/ml of alcalase hydrolysate (MW < 10 kDa). Crude protein have glutathione peroxidase activity less than MW < 10 kDa of protein hydrolysate.

Table 14 Crude protein and MW < 10 kDa of protein hydrolysate from spotted babylon prepared by alcalase, flavourzyme and neutrase. was measured unit/ml of glutathione peroxidase activity

Enzyme	GPx (unit/ml)	
	Crude protein	<10kDa
Alcalase	2.9±0.20[a]	5.8625±2.31[a]
Flavourzyme	0.7375±0.73[b]	3.6±1.41[b].
Neutrase	2.4875±0.94[a]	6.2625±0.93[a]
Control	0.6375±1.01[b]	3.475±1.98[b]

4.7 Gel filtration chromatography

As shown in Figure 12 and Table 15, flavourzyme hydrolysate (MW < 10 kDa) was divided into 3 subfractions including F1, F2 and F3. Among the 3 subfractions, F3 fraction exhibited the good scavenging activity on DPPH radical, ABTS radical and nitric oxide with IC_{50} values of 0.0075 ± 0.0021, 0.008 ± 0.0027 and 0.004 ± 0.001 µg/ml, respectively. Then, F1 and F2 have specifically IC_{50} values of ABTS radical scavenging activity which possess 0.023 ± 0.007 and 0.011 ± 0.002 µg/ml, respectively, but DPPH radical scavenging activity and nitric oxide scavenging activity were not show results due to too little concentration of protein was not calculated IC_{50} values. The results of purification by gel filtration chromatography found small protein molecule which was better result than large protein molecule. The same as Bougatef et al. (2010) reports the smallest molecule yielded the best results and Wang et al. (2013) reports that high peak was provided the good effect because there are many amino acid.

Table 15 The antioxidant activities of fractions from a column of Sephacryl S-200 gel filtration chromatography

Activity assay	IC_{50} (µg/ml)		
	F1	F2	F3
DPPH	> 0.058	> 0.052	0.0075 ± 0.0021
ABTS	0.023 ± 0.007	0.011 ± 0.002	0.008 ± 0.0027
NO	> 0.058	> 0.052	0.004 ± 0.001

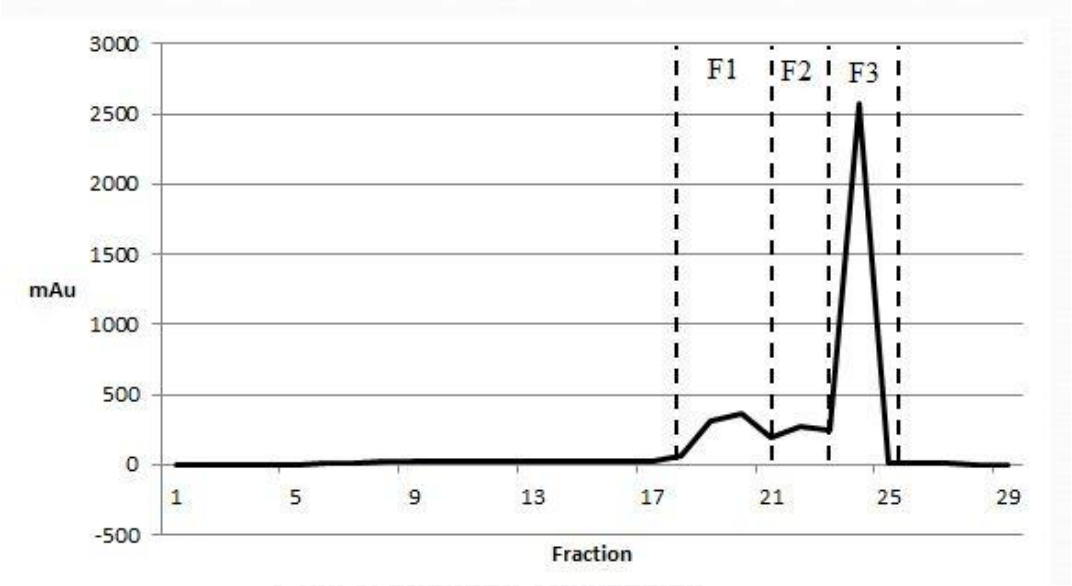


Figure 12 Chromatogram of flavourzyme hydrolysate protein (MW < 10 kDa) from Sephacryl S-200 gel filtration chromatography. Each fraction of the eluted solution was monitored at 280 nm.

4.8 Effects of spotted babylon hydrolysate on NO production in RAW 264.7 cells

The measurement of inhibited effect from spotted babylon hydrolysate on NO production and then RAW 264.7 cells were incubated with LPS (1 µg/ml) for overnight. The NO production in the culture medium was investigated by determination of the reduction of nitric oxide which is a stable metabolite of NO. As shown in Figure 13, the NO production of spotted babylon hydrolysate groups was

123.19 %, 281.02 %, 319.41 %, 323.47 %, 289.83 %, 272.15 %, 245.62 %, 221.63 %, 200.46 %, 180.62 %, 174.80 % and 164.03 % at the concentrations of 20, 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, 0.02 and 0.01 μM, respectively. The No production of control was 100 %. The results of this experiment was show inhibition of spotted babylon hydrolysate that was not inhibited NO production, but it stimulates to increase nitric oxide.

4.9 Cell viability in Raw 264.7 cells

The cytotoxic effect of spotted babylon hydrolysate in RAW 264.7 cells by using MTT assays. As shown in Figure 13, The control (treatment of LPS (1 μg/ml) alone) showed cytotoxic effect to RAW 264.7 cells which have differences between difference concentration of spotted babylon hydrolysate with control. When the macrophages cells were treated with 20, 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, 0.02 and 0.01 μM of spotted babylon hydrolysate which were found low cell viability is 36.71 % at concentration 20 μM and highest cell viability is 95.98 % at concentration 0.01 μM. The results indicated that up to the concentration about 0.63-0.01 μM of spotted babylon hydrolysate that did not affect RAW 264.7 cells. Combination the results of cell viability and NO production inhibitory effects was not exhibited the inhibitory effect of spotted babylon hydrolysate on NO production.

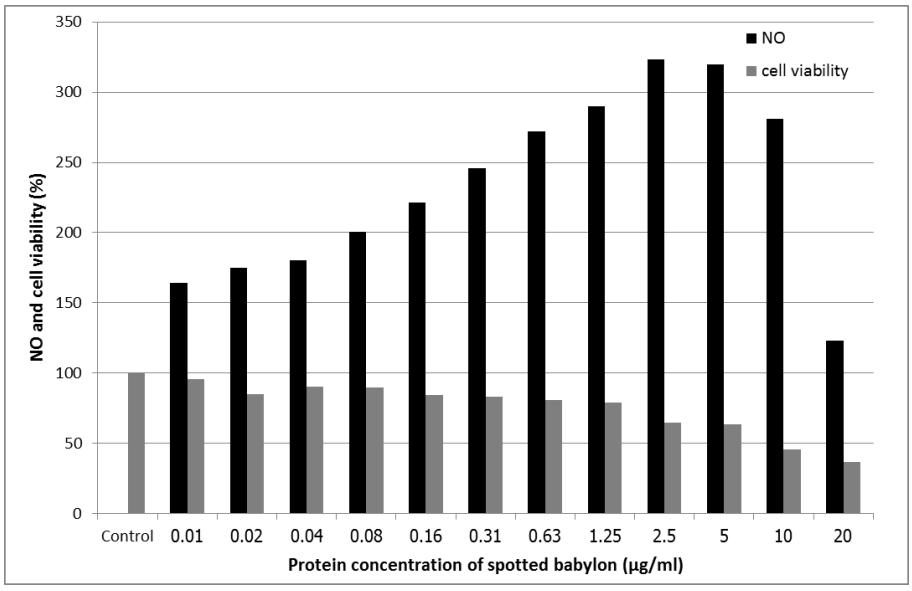


Figure 13 Inhibitory effect of spotted babylon hydrolysate on NO production in LPS stimulated RAW 264.7 cells and effect of spotted babylon hydrolysate on cell viability in RAW 264.7 cells.

4.10 RP-HPLC

The peptide fraction with the most active activity was further purified by reverse phase HPLC on a Shimpack C-18 column (250x46 mm) column with a linear gradient of acetonitrile 0.05% containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.7 ml/min. The eluted peaks were monitored at 280 nm. As shown in Figure 14, The result showed 4 purified peaks. These peaks were collected for further characterization by mass spectrometer.

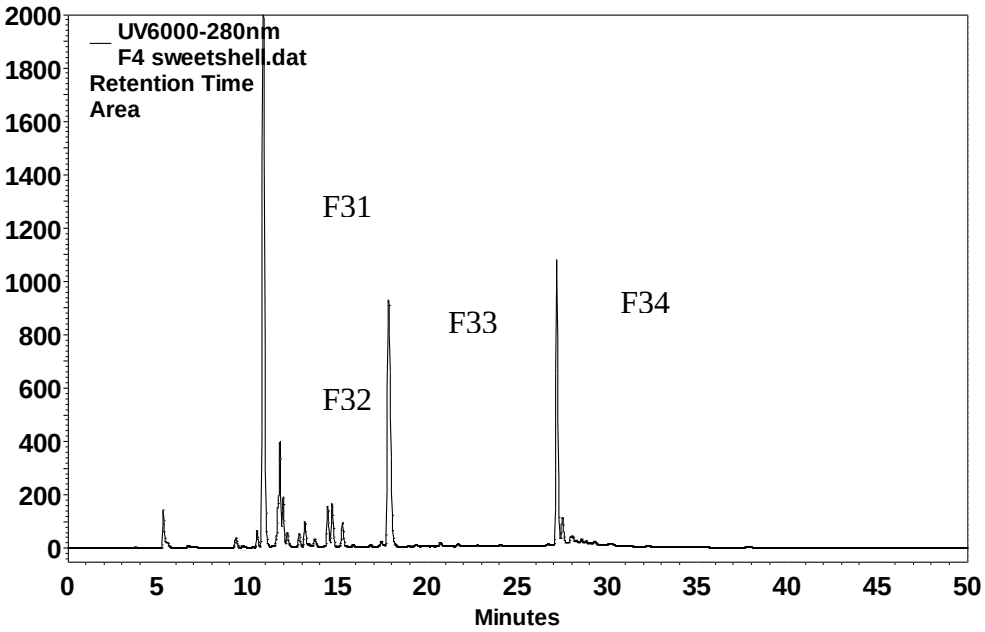


Figure 14 Chromatogram of fraction 4 from protein hydrolysate of spotted babylon (UV 280)

4.11 Mass spectrometry

The 4 bioactive peaks from RP-HPLC characterized by LC-MS/MS Q-TOF mass spectrometer. All peaks showed peptide sequence. In Table 16 showed 2 peptide chains of protein hydrolysate from peak 1. Peptide chains of protein hydrolysate

from peak 2, 3 and 4 showed in Table 17, 18 and 19, respectively. All peak had peptides that contain hydrophobic amino acid such as Pro, Leu, Ala, Trp and Phe, also show the highest antioxidant activity (Mendis, Rajapakse et al. 2005). In peak 1, 2 and 3, peptide chains contain aromatic amino acid Phe (F), Trp (W) and Tyr (Y) such as HTYHEVTKH (peak1) had Tyr (Y), WPVLAYHFT (peak2) had Phe (F), Trp (W) and Tyr (Y) and FAGRCKSTVE (peak3) contain Phe (F).

Hasegawa and co-worker (2012) study cysteine, histidine and glycine exhibit anti-inflammatory effects in human coronary arterial endothelial cells and Hasegawa and co-worker (2011)study exhibit anti-inflammatory effects in human monocytic leukemia cell line, THP-1 cells, there reports showed amino acids as Cys (C), His (H) and gly (G) possess anti-inflammation. Also, all peptide chains of protein hydrolysate contain amino acid (Cys(C), His(H) and gly (G) that show anti-inflammation. So, this purified peptide exhibited antioxidant and anti-inflammatory activity.

Table 16 Peptide sequence of peak 1 identified by LC-MS/MS Q-TOF

Sequence	Organism	Mass	Query cover	Identity	Accession
HTYHEVTKH	hypothetical protein LOTGIDRAFT_233146 [*Lottia gigantean*]	1151.65	55%	100%	XP_009056942.1
	hypothetical protein OCBIM_22012557mg [*Octopus bimaculoides*]	1151.65	55%	100%	KOF89811.1
	PREDICTED: myb-like protein W [Biomphalaria glabrata]	1151.65	55%	100%	XP_013080215.1
	Pre-mRNA-splicing factor 38B [*Crassostrea gigas*]	1151.65	55%	100%	EKC31902.1
	PREDICTED: ras-related protein Rab-18A isoform X2 [*Aplysia californica*]	1151.65	55%	100%	XP_005089064.1
PSLDATVPS PVSAHA	cytochrome c oxidase subunit II (mitochondrion)	1448.83	26%	100%	AII23638.1

	[*Babylonia areolata*]				
	cytochrome c oxidase subunit II (mitochondrion) [*Babylonia areolata*]	1448.83	26%	100%	YP_008963244.1
	NADH dehydrogenase subunit 5 (mitochondrion) [*Babylonia areolata*]	1448.83	40%	83%	AII23645.1
	NADH dehydrogenase subunit 5 (mitochondrion) [*Babylonia areolata*]	1448.83	40%	83%	YP_008963252.1
	Histone H3, partial [*Babylonia areolata*]	1448.83	26%	75%	AEP14878.1

Table 17 Peptide sequence of peak 2 identified by LC-MS/MS Q-TOF

Sequence	Organism	Mass	Query cover	Identity	Accession
WPVLAYHFT	cytochrome oxidase subunit I, partial (mitochondrion) [*Babylonia areolata*]	1133.64	44%	100%	ABS30401.1
	cytochrome oxidase subunit I, partial (mitochondrion) [*Babylonia areolata*]	1133.64	44%	100%	ABS30400.1
	cytochrome oxidase subunit I, partial (mitochondrion) [*Babylonia areolata*]	1133.64	44%	100%	ABS30390.1
	cytochrome b (mitochondrion) [*Babylonia areolata*]	1133.64	66%	67%	AII23642.1

Table 18 Peptide sequence of peak 3 identified by LC-MS/MS Q-TOF

Sequence	Organism	Mass	Query cover	Identity	Accession
RGSTSSSSRLN	NADH dehydrogenase subunit 4 (mitochondrion) [*Babylonia areolata*]	1151.65	45%	100%	AII23644.1
	NADH dehydrogenase subunit 5 (mitochondrion) [*Babylonia areolata*]	1151.65	81%	67%	AII23645.1
	ATP synthetase F0 subunit 8 (mitochondrion) [*Babylonia areolata*]	1151.65	54%	67%	AII23639.1
	NADH dehydrogenase subunit 6 (mitochondrion) [*Babylonia areolata*]	1151.65	18%	100%	AII23641.1
FAGRCKSTVE PVVKP	uncharacterized protein LOC106056025 [*Biomphalaria glabrata*]	1617.97	52%	100%	XP_013068028.1
	PREDICTED: uncharacterized protein LOC101857057 [*Aplysia californica*]	1617.97	40%	100%	XP_012943224.1
	Neurogenic locus notch-like protein 1 [*Crassostrea gigas*]	1617.97	40%	100%	EKC22858.1
	hypothetical protein LOTGIDRAFT_230032 [*Lottia gigantea*]	1617.97	40%	100%	XP_009044487.1
	hypothetical protein LOTGIDRAFT_158016 [*Lottia gigantea*]	1617.97	73%	60%	XP_009048841.1

Table 19 Peptide sequence of Peak 4 identified by LC-MS/MS Q-TOF

Sequence	Organism	Mass	Query cover	Identity	Accession
RPSPSSLPGKH	ATP synthetase F0 subunit 6 (mitochondrion) [*Babylonia lutosa*]	1162.69	54%	55%	AHH30668.1
	ATP synthase F0 subunit 6 (mitochondrion) [*Babylonia lutosa*]	1162.69	54%	55%	ADU77469.1
	NADH dehydrogenase subunit 2 (mitochondrion) [*Babylonia areolata*]	1162.69	36%	100%	AII23636.1
	NADH dehydrogenase subunit 2 (mitochondrion) [*Babylonia lutosa*]	1162.69	36%	100%	AHH30665.1
	NADH dehydrogenase subunit 4L (mitochondrion) [*Babylonia lutosa*]	1162.69	18%	100%	ADU77472.1

CHAPTER V

CONCLUSION

In this study, the best antioxidant activity peptide derived from spotted babylon was hydrolysed using flavozyme hydrolysate compared to other enzymes by 2 testing. The first assay is non-enzymatic antioxidant by using DPPH, ABTS, and nitric oxide scavenging. Then, superoxide radical scavenging activity assay, catalase activity assay and glutathione peroxidase activity assay are enzymatic antioxidant of the second assay. Therefore, F3 fraction was purified by ultrafiltration (MW < 10 kDa) and gel filtration chromatography. 3 fractions were obtained from gel filtration chromatography. Thus, F3 fraction of gel filtration chromatography purification is the best fraction which was purified to RP-HPLC and mass spectrometry. RP-HPLC harvests 4 fraction that purified by mass spectrometry was obtained 6 sequence in 4 peak. After that, F3 fraction from gel filtration chromatography was tested in RAW 264.7 cells which are macrophage by inhibition of NO production assay and MTT assay. The results of cell viability and NO production inhibitory effects was not exhibited the inhibitory effect of spotted babylon hydrolysate on NO production.

REFERENCES

Ahn, C.-B., Y.-S. Cho and J.-Y. Je (2015). "Purification and anti-inflammatory action of tripeptide from salmon pectoral fin byproduct protein hydrolysate." Food Chemistry **168**: 151-156.

Ahn, C.-B., J.-Y. Je and Y.-S. Cho (2012). "Antioxidant and anti-inflammatory peptide fraction from salmon byproduct protein hydrolysates by peptic hydrolysis." Food Research International **49**(1): 92-98.

Bauchart, C., C. Chambon, P. P. Mirand, I. Savary-Auzeloux, D. Rémond and M. Morzel (2007). "Peptides in rainbow trout (Oncorhynchus mykiss) muscle subjected to ice storage and cooking." Food Chemistry **100**(4): 1566-1572.

Bendich, A., L. J. Machlin, O. Scandurra, G. W. Burton and D. D. M. Wayner (1986). "The antioxidant role of vitamin C." Advances in Free Radical Biology & Medicine **2**(2): 419-444.

Bougatef, Ali, A. Nedjar, Naima, Manni, Laïla, Ravallec, Rozenn, Barkia, Ahmed, Guillochon, Didier, Nasri and Moncef (2010). Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (Sardinella aurita) by-products proteins, Elsevier Ltd.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Analytical Biochemistry **72**(1): 248-254.

Brigelius-Flohé, R. (1999). "Tissue-specific functions of individual glutathione peroxidases." Free Radical Biology and Medicine **27**(9–10): 951-965.

Cai, Y., Q. Luo, M. Sun and H. Corke (2004). "Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer." Life Sciences **74**(17): 2157-2184.

Calder, P. C., R. Albers, J. M. Antoine, S. Blum, R. Bourdet-Sicard, G. A. Ferns, G. Folkerts, P. S. Friedmann, G. S. Frost, F. Guarner, M. Lovik, S. Macfarlane, P. D. Meyer, L. M'Rabet, M. Serafini, W. van Eden, J. van Loo, W. Vas Dias, S. Vidry, B. M. Winklhofer-Roob and J. Zhao (2009). "Inflammatory disease

processes and interactions with nutrition." British Journal of Nutrition **101 Suppl 1**: S1-45.

Chaitanawisuti, N. and A. Kritsanapuntu (1999). "Growth and production of hatchery-reared juvenile spotted babylon Babylonia areolata Link, 1807 cultured to marketable sizes in intensive flow-through and semi-closed recirculating water system." Aquaculture Research 31: 415-419.

Chantaranothai, C., T. Palaga, A. Karnchanatat and P. Sangvanich (2013). "Inhibition of nitric oxide production in the macrophage-like RAW 264.7 cell line by protein from the rhizomes of Zingiberaceae plants." Preparative Biochemistry and Biotechnology **43**(1): 60-78.

Chelikani, P., I. Fita and P. C. Loewen (2004). "Diversity of structures and properties among catalases." Cellular and Molecular Life Sciences CMLS **61**(2): 192-208.

Cheng, M.-L., H.-C. Wang, K.-C. Hsu and J.-S. Hwang (2015). "Anti-inflammatory peptides from enzymatic hydrolysates of tuna cooking juice." Food and Agricultural Immunology **26**(6): 770-781.

Ciruelos, E., J. M. Trigo, B. Pardo, L. Paz-Ares, N. Estaun, C. Cuadra, M. Dominguez, A. Marin, J. Jimeno and M. Izquierdo (2002). "A phase I clinical and pharmacokinetic (PK) study with Kahalalide F (KF) in patients (pts) with advanced solid tumors (AST) with a continuous weekly (W) 1-hour ivinfusion schedule." European Journal of Cancer **38**: 33.

Dávalos, A., M. Miguel, B. Bartolomé and R. López-Fandiño (2004). "Antioxidant Activity of Peptides Derived from Egg White Proteins by Enzymatic Hydrolysis." Journal of Food Protection **67**(9): 1939-1944.

Gordon, M. H. (1990). The Mechanism of Antioxidant Action in Vitro. Food Antioxidants. B. J. F. Hudson, Springer Netherlands**:** 1-18.

Govindarajan, R., S. Rastogi, M. Vijayakumar, A. Shirwaikar, A. K. Rawat, S. Mehrotra and P. Pushpangadan (2003). "Studies on the antioxidant activities of Desmodium gangeticum." Biological and Pharmaceutical Bulletin **26**(10): 1424-1427.

Hanada, T. and A. Yoshimura (2002). "Regulation of cytokine signaling and inflammation." Cytokine and Growth Factor Reviews **13**(4): 413-421.

Hauton, C., V. Brockton and V. J. Smith (2006). "Cloning of a crustin-like, single whey-acidic-domain, antibacterial peptide from the haemocytes of the European lobster, *Homarus gammarus*, and its response to infection with bacteria." Molecular Immunology **43**(9): 1490-1496.

Heo, S. J., Y. J. Jeon, J. Lee, H. T. Kim and K. W. Lee (2003). "Antioxidant effect of enzymatic hydrolyzate from a kelp, Ecklonia cava. ." Algae **18**: 341-347.

Je, J.-Y., P.-J. Park, H.-G. Byun, W.-K. Jung and S.-K. Kim (2005). "Angiotensin I converting enzyme (ACE) inhibitory peptide derived from the sauce of fermented blue mussel, *Mytilus edulis.*" Bioresource Technology **96**(14): 1624-1629.

Jiang, H., T. Tong, J. Sun, Y. Xu, Z. Zhao and D. Liao (2014). "Purification and characterization of antioxidative peptides from round scad (*Decapterus maruadsi*) muscle protein hydrolysate." Food Chemistry **154**: 158-163.

Jung, W.-K. and S.-K. Kim (2009). "Isolation and characterisation of an anticoagulant oligopeptide from blue mussel, Mytilus edulis." Food Chemistry **117**(4): 687-692.

Katano, S., T. Oki, Y. Matsuo, K. Yoshihira, Y. Nara, T. Miki, T. Matsui and K. Matsumoto (2003). "Antihypertensive effect of alkaline protease hydrolysate of the pearl oyster Pinctada fucata martencii & separation and identification of angiotensin-I converting enzyme inhibitory peptides." NIPPON SUISAN GAKKAISHI **69**(6): 975-980.

Kim, E.-K., Y.-S. Kim, J.-W. Hwang, S. H. Kang, D.-K. Choi, K.-H. Lee, J. S. Lee, S.-H. Moon, B.-T. Jeon and P.-J. Park (2013). "Purification of a novel nitric oxide inhibitory peptide derived from enzymatic hydrolysates of Mytilus coruscus." Fish & Shellfish Immunology **34**(6): 1416-1420.

Kim, E.-K., S.-J. Lee, B.-T. Jeon, S.-H. Moon, B. Kim, T.-K. Park, J.-S. Han and P.-J. Park (2009). "Purification and characterisation of antioxidative peptides from

enzymatic hydrolysates of venison protein." Food Chemistry **114**(4): 1365-1370.

Lee, J. M., S. G. You and S. M. Kim (2005). "Functional activities of low molecular weight peptides purifiedfrom enzymatic hydrolysates of seaweeds." The Journal of the Korean Society of Food Science and Nutrition **34**: 1124-1129.

Lee, T.-G. and S. Maruyama (1998). "Isolation of HIV-1 Protease-Inhibiting Peptides from Thermolysin Hydrolysate of Oyster Proteins." Biochemical and Biophysical Research Communications **253**(3): 604-608.

Lin, L. and B.-f. Li (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**(14): 2290-2295.

Liu, Z., S. Dong, J. Xu, M. Zeng, H. Song and Y. Zhao (2008). "Production of cysteine-rich antimicrobial

peptide by digestion of oyster (Crassostrea gigas) with alcalase and bromelin." Food Control **19**: 231-235

Lobo, V., A. Patil, A. Phatak and N. Chandra (2010). "Free radicals, antioxidants and functional foods: Impact on human health." Pharmacognosy Reviews **4**(8): 118-126.

Lü, J.-M., P. H. Lin, Q. Yao and C. Chen (2010). "Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems." Journal of Cellular and Molecular Medicine **14**(4): 840-860.

Madden, T., H. T. Tran, D. Beck, R. Huie, R. A. Newman, L. Pusztai, J. J. Wright and J. L. Abbruzzese (2000). "Novel Marine-derived Anticancer Agents: A Phase I Clinical, Pharmacological, and Pharmacodynamic Study of Dolastatin 10 (NSC 376128) in Patients with Advanced Solid Tumors." Clinical Cancer Research **6**(4): 1293-1301.

Masaaki, Y. and F. Hiroyuki (2007). Marine-Derived Protein Hydrolysates, Their Biological Activities and Potential as Functional Food Ingredients. Marine Nutraceuticals and Functional Foods, CRC Press**:** 247-258.

Masuda, M., H. Nakashima, T. Ueda, H. Naba, R. Ikoma, A. Otaka, Y. Terakawa, H. Tamamura, T. Ibuka, T. Murakami, Y. Koyanagi, M. Waki, A. Matsumoto, N. Yamamoto, S. Funakoshi and N. Fujii (1992). "A novel anti-HIV synthetic peptide, T-22 ([Tyr5,12,Lys7]-polyphemusin II)." Biochemical and Biophysical Research Communications **189**(2): 845-850.

Mendis, E., N. Rajapakse and S.-K. Kim (2005). "Antioxidant Properties of a Radical-Scavenging Peptide Purified from Enzymatically Prepared Fish Skin Gelatin Hydrolysate." Journal of Agricultural and Food Chemistry **53**(3): 581-587.

Mitta, G., F. Hubert, T. Noël and P. Roch (1999). "Myticin, a novel cysteine-rich antimicrobial peptide isolated from haemocytes and plasma of the mussel Mytilus galloprovincialis." European Journal of Biochemistry **265**(1): 71-78.

Mohsen, S. M. and A. S. M. Ammar (2009). "Total phenolic contents and antioxidant activity of corn tassel extracts." Food Chemistry **112**(3): 595-598.

Moure, A., J. M. Cruz, D. Franco, J. M. Domínguez, J. Sineiro, H. Domínguez, M. a. José Núñez and J. C. Parajó (2001). "Natural antioxidants from residual sources." Food Chemistry **72**(2): 145-171.

Ngo, D.-H., B. Ryu, T.-S. Vo, S. W. A. Himaya, I. Wijesekara and S.-K. Kim (2011). "Free radical scavenging and angiotensin-I converting enzyme inhibitory peptides from Pacific cod (Gadus macrocephalus) skin gelatin." International Journal of Biological Macromolecules **49**(5): 1110-1116.

Park, I. Y., C. B. Park, M. S. Kim and S. C. Kim (1998). "Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, Parasilurus asotus." FEBS Letters **437**(3): 258-262.

Pham-Huy, L. A., H. He and C. Pham-Huy (2008). "Free Radicals, Antioxidants in Disease and Health." International Journal of Biomedical Science : IJBS **4**(2): 89-96.

Pokora, M., E. Eckert, A. Zambrowicz, Ł. Bobak, M. Szołtysik, A. Dąbrowska, J. Chrzanowska, A. Polanowski and T. Trziszka (2013). "Biological and

functional properties of proteolytic enzyme-modified egg protein by-products." Food Science & Nutrition **1**(2): 184-195.

Prakot, P., N. Chaitanawisuti and A. Karnchanatat (2014). "Anti-tyrosinase activity of protein hydrolysate from spotted babylon Babylonia areolata prepared by alkaline protease." thesis: 25-39.

Qian, Z.-J., W.-K. Jung, H.-G. Byun and S.-K. Kim (2008). "Protective effect of an antioxidative peptide purified from gastrointestinal digests of oyster, Crassostrea gigas against free radical induced DNA damage." Bioresource Technology **99**(9): 3365-3371.

Rajapakse, N., E. Mendis, H.-G. Byun and S.-K. Kim (2005). "Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems." The Journal of Nutritional Biochemistry **16**(9): 562-569.

Rajapakse, N., E. Mendis, W.-K. Jung, J.-Y. Je and S.-K. Kim (2005). "Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties." Food Research International **38**(2): 175-182.

Saito, M. and H. Hagino (2005). "Antihypertensive effect of oligopeptides derived from nori *(Porphyra yezoensis)* and Ala-Lys-Tyr-Ser-Tyr in rats." Journal of Japanese Society of Nutrition and Food Science **58**: 177-184.

Samaranayaka, A. G. P. and E. C. Y. Li-Chan (2011). "Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications." Journal of Functional Foods **3**(4): 229-254.

Shahidi, F. and Y. Zhong (2010). "Novel antioxidants in food quality preservation and health promotion." European Journal of Lipid Science and Technology **112**(9): 930-940.

Shang-gui, D., P. Zhi-ying, C. Fang, Y. Ping and W. Tie (2004). "Amino acid composition and anti-anaemia action of hydrolyzed offal protein from Harengula Zunasi Bleeker." Food Chemistry **87**(1): 97-102.

Shigeoka, S., T. Ishikawa, M. Tamoi, Y. Miyagawa, T. Takeda, Y. Yabuta and K. Yoshimura (2002). "Regulation and function of ascorbate peroxidase isoenzymes." Journal of Experimental Botany **53**(372): 1305-1319.

Suetsuna, K. (2000). "Antioxidant Peptides from the Protease Digest of Prawn (Penaeus japonicus) Muscle." Marine Biotechnology **2**(1): 5-10.

Taherzadeh, M. J. and K. Karimi (2007). "Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials: a review." Bioresources *2*: 707-738.

Traber, M. G. and J. Atkinson (2007). "Vitamin E, Antioxidant and Nothing More." Free radical biology & medicine **43**(1): 4-15.

Tsai, J.-S., J.-L. Chen and B. S. Pan (2008). "ACE-inhibitory peptides identified from the muscle protein hydrolysate of hard clam (Meretrix lusoria)." Process Biochemistry **43**(7): 743-747.

Valko, M., D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur and J. Telser (2007). "Free radicals and antioxidants in normal physiological functions and human disease." The International Journal of Biochemistry & Cell Biology **39**(1): 44-84.

Yang, X., M. C. Kang, Y. Li, E. A. Kim, S. M. Kang and Y. J. Jeon (2014). "Anti-inflammatory activity of questinol isolated from marine-derived fungus Eurotium amstelodami in lipopolysaccharide-stimulated RAW 264.7 macrophages." J Microbiol Biotechnol **24**(10): 1346-1353.

Zelko, I. N., T. J. Mariani and R. J. Folz (2002). "Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression." Free Radical Biology and Medicine **33**(3): 337-349.

APPENDIX

APPENDIX A

Preparation of buffer and reagents

1. 20 mM Phosphate buffer pH 7.2

20mM KH_2PO_4	2.72 g
20mM K_2HPO_4	8.71 g
150mM NaCl	8.77 g
Deionized water	1000 ml

Protocal: Mix 1000 ml deionized water with all reagents. Adjust the pH to 7.2 with KOH

2. Bradford solution

- Bradford stock solution

95% Ethanol	100 ml
88% Phosphoric acid	200 ml
SERVA Blue G	350 g

- Bradford working buffer

Deionized water	425 ml
95% Ethanol	15 ml
88% Phosphoric acid	30 ml
Bradford stock solution	30 ml

Note: Before using, Bradford working buffer must be filtered through the Whatman No.1 paper. It is stored in a brown glass bottle at room temperature.

Protocal: 1. Pipet protein solution into 96 well plates 20 µl.

2. Add Bradford working buffer 200 µl and shake.

3. Read A_{595} after 2 minutes but before 1 hour.

3. DPPH solution

0.1 M DPPH	0.004 mg
Methanol	100 ml

Protocal: 1. Pipet protein solution into 96 well plates 40 µl.

2. Add DPPH solution 160 µl and incubate 20 min in the dark room.

3. Shake and Read the absorbance at 517 nm.

4. ABTS solution

- 7 mM ABTS (solution A)

Dissolve 0.096 g ABTS in 25 ml deionized water.

- 2.45 mM potassium persulphate (solution B).

Dissolve 0.016 g potassium persulphate in 25 ml deionized water

- ABTS solution

Mix solution A and solution B in the dark room for 12 – 16 hours before using. Before use it, dilute ABTS solution with distilled water to obtain an absorbance value of 0.7 ± 0.02 at 734 nm

Protocal: 1. Pipet protein solution into 96 well plates 10 µl.

2. Add ABTS solution 300 µl and incubate 10 min.

3. Shake and Read the absorbance at 734 nm

5. Nitric oxide method

- 0.1% (w/v) N- (1-Napthyl) ethylenediamine dihydrochloride (NED)

Dissolve 0.1 g NED in 100ml deionized water.

- 10 mM sodium nitroprusside (SND) in PBS pH 7.2

Dissolve 0.29 g Sodium nitroprusside in 100 ml Phosphate buffer pH 7.2

- 0.33% (w/v) sulfanilamide in 20% acetic acid

Start with dissolve 0.33 g sulfanilamide in 100 ml 20% acetic acid (20 ml acetic in 80 ml distilled water.

Protocal : 1. Pipet protein solution into 96 well plates 25 µl.

2. Add SNP 25 µl and incubate 2.5 h

3. Add 0.33% (w/v) sulfanilamide 100 µl and incubate 5 min

4. Add 0.1% NED 100 µl and incubate 30 min

5. Shake and read the absorbance at 540 nm

6. Media: Complete DMEM

DMEM	100%
Fetal Bovine Serum (FBS)	10%

7. MTT solution

5 mg/ml MTT solution

MTT	5 mg
Deionized water	1 ml

Protocal: dissolve 5 mg MTT with 1 ml deionized water.

8. LPS 1 µg/ml

LPS 500 µg/ml	4 µl
DMEM	2 ml

9. Mobile phase in RP-HPLC analysis

- Eluent A: 0.1% trifluoroacetic acid (TFA), 1000 ml

start with add 1 ml TFA into 999 ml double deionized water followed by filtration using a cellulose acetate membrane.

- Eluent B: 70 % acetonitrile containing 0.05% TFA, 1000 ml

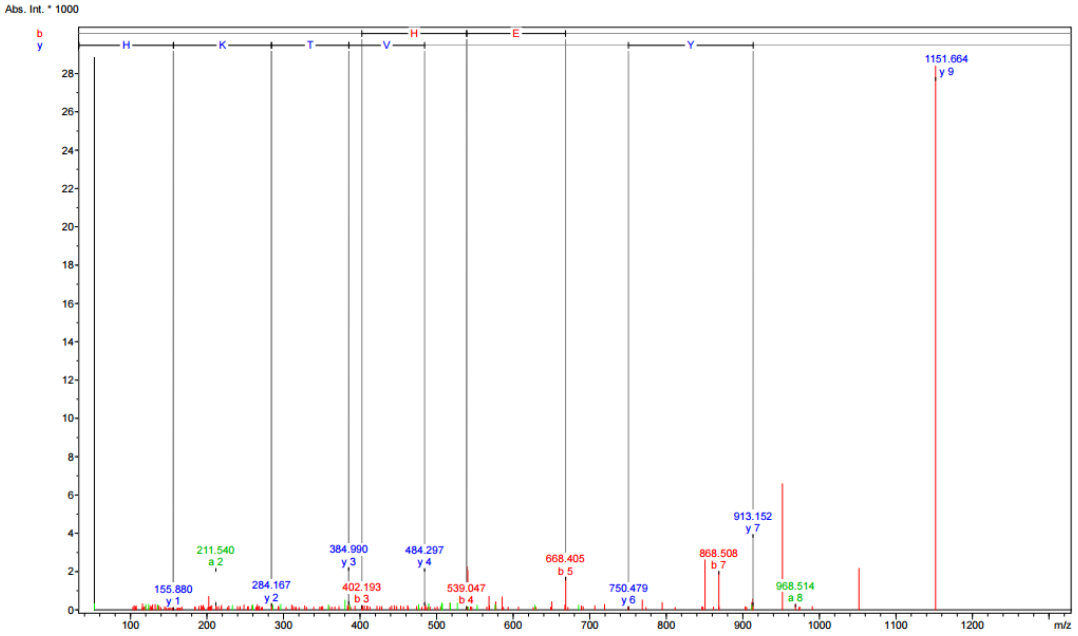
start with adding 300 ml 0.05% TFA in double deionized water into 700 ml 70% acetonitrile and mixing followed by filtration using PTFE membrane.

APPENDIX B

PEAK1

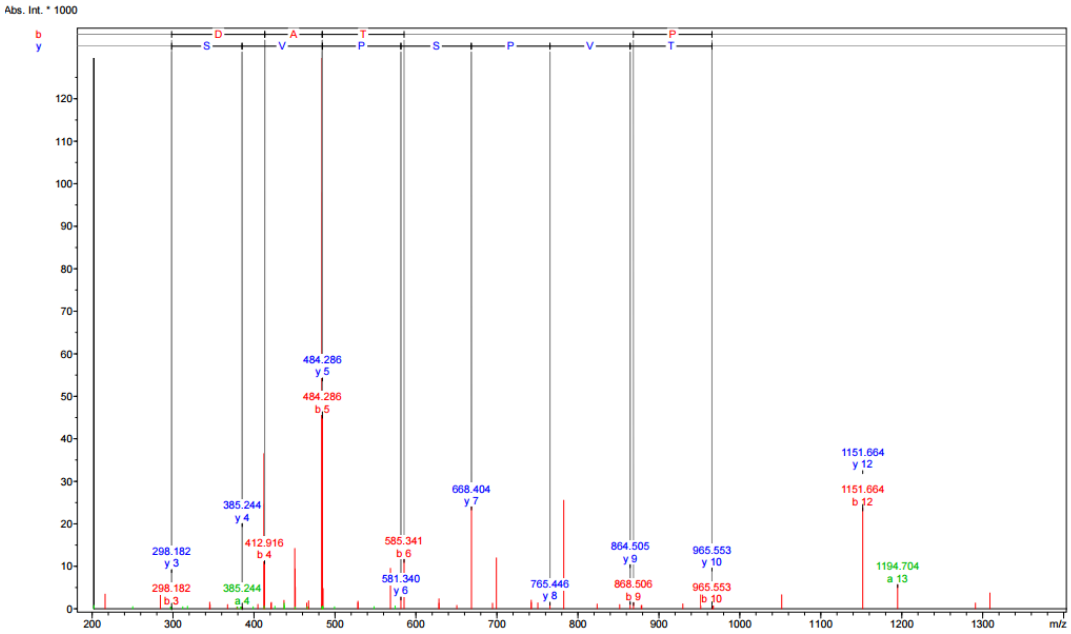
Sequence 1: .HTYHEVTKH

				Parentmass:	1151.658
Mass Error:	0.098	MH+ (mono):	1151.560	MH+ (avg):	1152.242
Threshold (a.i.):	0.000	Tolerance (Da):	0.500	Number of Peaks:	201
Above Threshold:	201	Assigned Peaks:	13	Not assigned Peaks:	188



Sequence 2: PSLDATVPSPVSAHA

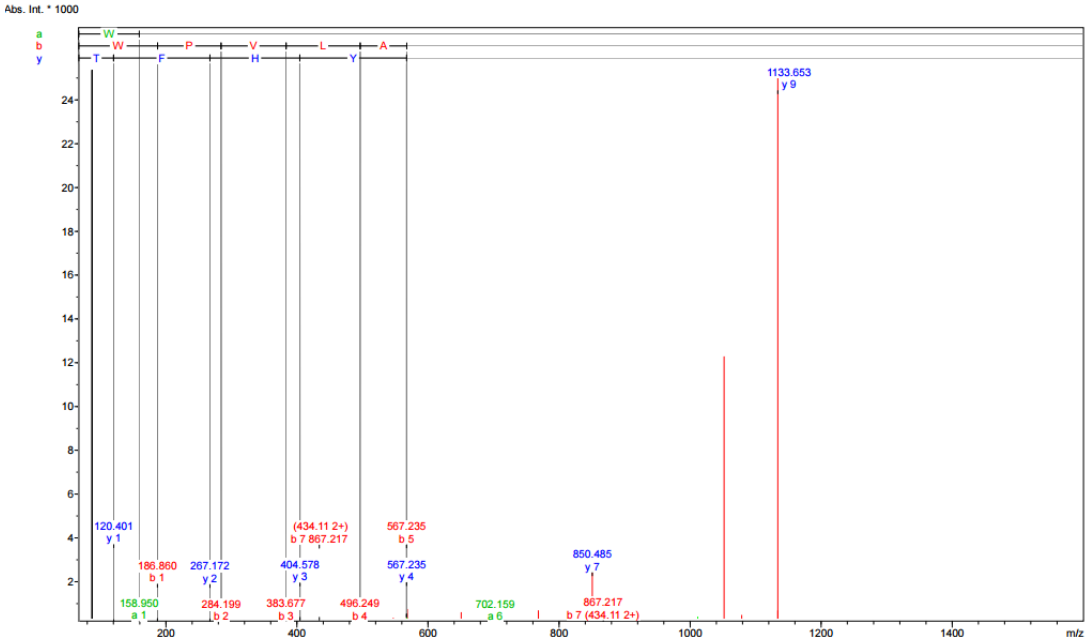
				Parentmass:	1448.837
Mass Error:	0.098	MH+ (mono):	1448.739	MH+ (avg):	1449.588
Threshold (a.i.):	0.000	Tolerance (Da):	0.500	Number of Peaks:	87
Above Threshold:	87	Assigned Peaks:	13	Not assigned Peaks:	74



PEAK2

Sequence 1: WPVLAYHFT

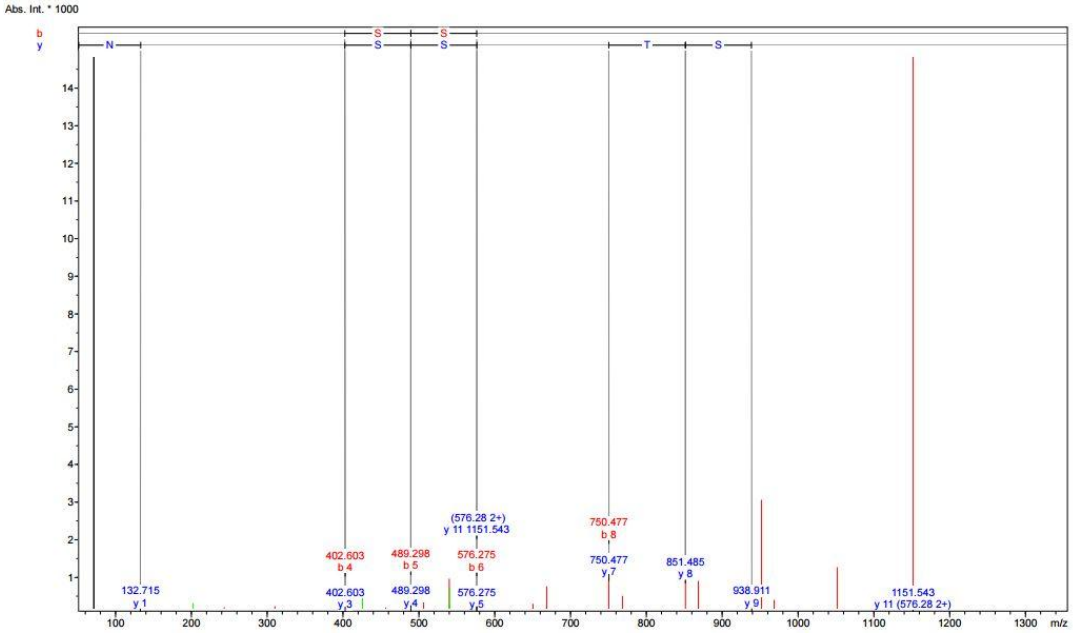
		Parentmass: 1133.649
Mass Error: 0.071	MH+ (mono): 1133.578	MH+ (avg): 1134.308
Threshold (a.i.): 0.000	Tolerance (Da): 0.500	Number of Peaks: 161
Above Threshold: 161	Assigned Peaks: 13	Not assigned Peaks: 148



PEAK3

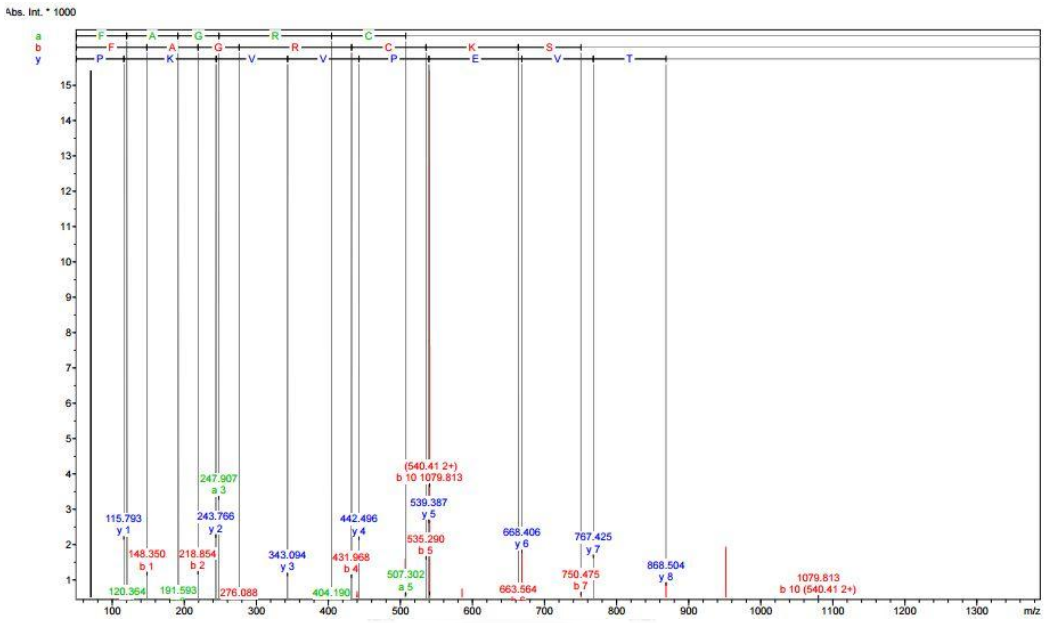
Sequence 1: RGSTSSSSRLN

				Parentmass:	1151.659
Mass Error:	0.082	MH+ (mono):	1151.577	MH+ (avg):	1152.199
Threshold (a.i.):	0.000	Tolerance (Da):	0.500	Number of Peaks:	55
Above Threshold:	55	Assigned Peaks:	8	Not assigned Peaks:	47



Sequence 2: FAGRCKSTVEPVVKP

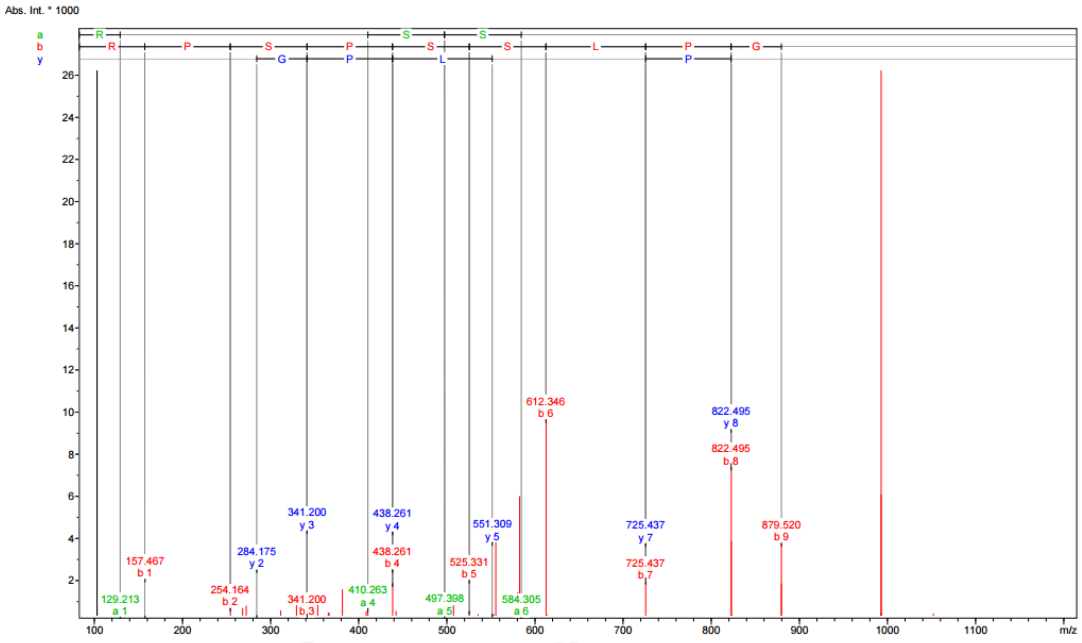
				Parentmass:	1617.975
Mass Error:	0.096	MH+ (mono):	1617.879	MH+ (avg):	1618.923
Threshold (a.i.):	0.000	Tolerance (Da):	0.500	Number of Peaks:	198
Above Threshold:	198	Assigned Peaks:	21	Not assigned Peaks:	177



PEAK4

Sequence 1: RPSPSSLPGKH

		Parentmass: 1162.691
Mass Error: 0.058	MH+ (mono): 1162.633	MH+ (avg): 1163.310
Threshold (a.i.): 0.000	Tolerance (Da): 0.500	Number of Peaks: 144
Above Threshold: 144	Assigned Peaks: 15	Not assigned Peaks: 129



VITA

Mister Komson Techoaphiwatanakun was born on June 6th, 1991 in Chaingmai, Thailand. He graduated with a Bachelor's degree of Science from Department of Bio production Technology, Faculty of Sciences and Industrial Technology, Prince of Songkhla University, Suratthani Campus in 2012. He had further studied to the Master Degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University in 2013.

Agricultural Science and Technology

Academic presentations;

Techoaphiwatanakun, K., Karnchanatat, A. and Chaitanawisuti, N. Antioxidation peptides derived from enzymatic hydrolysis of spotted Babylon (Babylonia arelata). The 10th International Symposium of the Protein Society of Thailand, 15-17th July 2015, Convention center, Chulabhorn Research Institute Bangkok, Thailand.