ฤทธิ์ขจัดอนุมูลอิสระและยับยั้งการเพิ่มจำนวนเซลล์มะเร็งของโปรตีนไฮโดรไลเสตจาก
หอยหวาน *Babylonia areolata* ที่เตรียมจากเพปซิน-แพนครีเอตินและปาเปน

นางสาวพัชชา เพ็ชรสันทัด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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FREE RADICAL SCAVENING AND ANTIPROLIFERATION OF PROTEIN HYDROLYSATE FROM SPOTTED BABYLON *Babylonia areolata* PREPARED BY PEPSIN-PANCREATIN AND PAPAIN

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	FREE RADICAL SCAVENING AND ANTIPROLIFERATION OF PROTEIN HYDROLYSATE FROM SPOTTED BABYLON *Babylonia areolata* PREPARED BY PEPSIN-PANCREATIN AND PAPAIN
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พัชชา เพ็ชรสันทัด : ฤทธิ์ขจัดอนุมูลอิสระและยับยั้งการเพิ่มจำนวนเซลล์มะเร็งของโปรตีนไฮโดรไลเสตจากหอยหวาน *Babylonia areolata* ที่เตรียมจากเพปซิน-แพนครีเอตินและปาเปน (FREE RADICAL SCAVENING AND ANTIPROLIFERATION OF PROTEIN HYDROLYSATE FROM SPOTTED BABYLON *Babylonia areolata* PREPARED BY PEPSIN-PANCREATIN AND PAPAIN) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.อภิชาติ กาญจนทัต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร.นิลนาจ ชัยธนาวิสุทธิ์, 70 หน้า.

ในปัจจุบันโลกมีสภาวะแวดล้อมที่มีมลพิษในปริมาณมาก ส่งผลต่อสุขภาพที่เสื่อมโทรม ซึ่งเป็นสาเหตุของการเกิดโรคต่าง ๆ เป็นจำนวนมาก โดยเฉพาะโรคมะเร็งซึ่งเป็นโรคที่อันตรายยากต่อการรักษา และเกิดผลข้างเคียงต่อผู้ป่วยเป็นจำนวนมาก ซึ่งต้นเหตุของโรค คือการที่ร่างกายมีอนุมูลอิสระมากเกินกว่าที่ร่างกายจะกำจัดหรือยับยั้งได้ ทำให้ไปทำลายโมเลกุล ดีเอ็นเอ เซลล์ เนื้อเยื่อ และอวัยวะต่าง ๆ ของร่างกาย เกิดการเสื่อมสภาพและนำไปสู่การเกิดโรคต่าง ๆ ดังนั้นจึงสรรหาสารที่มีความสามารถในการต้านอนุมูลอิสระ ดังนั้นงานวิจัยนี้มีจุดประสงค์ เพื่อศึกษาการเตรียมเปปไทด์จากโปรตีนไฮโดรไลเสตจากหอยหวานด้วยเอมไซม์โปรติเอสของพืชและจำลองระบบทางเดินอาหารของมนุษย์ที่มีฤทธิ์ในการต้านอนุมูลอิสระและนำโปรตีนไฮโดรไลเสตที่มีค่าการต้านอนุมูลอิสระสูงสุด(หอยหวาน 0.075 g/ml ที่ย่อยด้วยเอมไซม์เปปซินและแพนคริเอติน) มาผ่านกระบวนการอัลตราฟิลเตรชั่นซึ่งทำการแยกออกเป็น 5 ขนาดของน้ำหนักโมเลกุล จากนั้นนำมาทดสอบฤทธิ์การต้านอนุมูลอิสระด้วยทั้งหมด 3 วิธี คือฤทธิ์ยับยั้งอนุมูลอิสระด้วยสาร DPPH ABTS และ NO พบว่าที่ขนาดโมเลกุล 3 กิโลดาลตัน มีค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งอนุมูลอิสระได้ร้อยละ 50 ได้ต่ำที่สุดคือ 0.918 ± 0.097, 0.502±0.075 and 0.420±0.069 µg/ml ตามลำดับ จากนั้นนำไปทำให้บริสุทธิ์และระบุลักษณะของเปปไทด์จากโปรตีนไฮโดรไลเสตของหอยหวานได้ทั้งหมด 8 สาย นอกจากนี้ได้ทำการทดสอบฤทธิ์ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งจำนวน 5 ชนิด และทดสอบลักษณะการตายของเซลล์มะเร็ง พบว่าโปรตีนไฮโดรไลเสตขนาด 3 กิโลดาลตัน สามารถยับยั้งการเจริญเติบโตของเซลล์มะเร็งปอด (CHAGO-K1)ได้ดีที่สุดและมีลักษณะการตายของเซลล์แบบอะพอพโทซิสเพียงเล็กน้อย เมื่อเทียบกับด๊อกโซรูบีซิน ซึ่งเป็นยาต้านมะเร็งที่ใช้อยู่ในปัจจุบัน

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

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PUTCHA PETSANTAD: FREE RADICAL SCAVENING AND ANTIPROLIFERATION OF PROTEIN HYDROLYSATE FROM SPOTTED BABYLON *Babylonia areolata* PREPARED BY PEPSIN-PANCREATIN AND PAPAIN. ADVISOR: ASST. PROF. APHICHART KARNCHANATAT, Ph.D., CO-ADVISOR: NINNAJ CHAITANAWISUTI, Ph.D., 70 pp.

The world is a heavily polluted environment which affects health and is the cause of many diseases, especially cancer. Cancer is difficult to treat, and there are often adverse side effects for the patient. These diseases produce more free radicals than the body can inhibit which cause damage to molecules, DNA, cells, and body organs. Antioxidants in the body are beneficial for health. This research investigated the preparation of peptides from spotted babylon snails protein hydrolysates by plant proteases and the simulation of the human digestive system with the effect of antioxidant activity. The protein hydrolysate had the highest antioxidant activity (spotted babylon at 0.075 g/ml digested by pepsin-pancreatin); through a ultration system with five separate molecular weights. The antioxidant activity was tested by three methods, DPPH, ABTS, and NO radical scavenging assays. It was found that the molecular weight of protein 3 kDa had the highest inhibition antioxidant activity (IC_{50} values of 0.918 ± 0.097, 0.502±0.075 and 0.420±0.069 µg/ml respectively.). The purification and identification of characteristics from protein hydrolysates of spotted babylon showed eight peptides. In addition, results indicated inhibition in the growth of cancer cells for five species which were tested for apoptosis of cancer cells. It was found that protein hydrolysate at 3 kDa produced the highest antiproliferative of the growth of lung cancer cells (CHAGO-K1), with less apoptosis compared to doxorubicin which is an anticancer drug used in medicine.

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LIST OF ABBREVIATION

Ab	Absorbance
ml	Milliliter
l	Liter
µ	Microliter
g	Gram
℃	Degree celsius
mg	Milligram
mg/l	Milligram per liter
M	Molar
Mm	Mill molar
mmol	Mill mole
min	Min
hr	Hour
IC_{50}	Median inhibitory
Cone.	Concentration
w/v	Weight per volume
w/w	Weight per weight
v/v	Volume per volume
MWCO	Molecular weight
rpm	Revolution per min
NaCl	Sodium chloride
BSA	Bovine serum albumin
EDTA	Ethylenediamine tetraacetic acid
NED	N-(1-naphthyl) ethylene diamine
S.E.	Standard error
et al.	And others

%	Percentage
SOD	Superoxide dismutase
SODs	Superoxide dismutase enzymes
$O_2^{\bullet-}$	Superoxide anion
H_2O_2	Hydrogen peroxide
BHT	Butylated hydroxyl toluene
BHA	Butylated hydroxyl anisole
DPPH	1,1-Diphenyl-2-picrylhydrazyl
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
NO	Nitric oxide
MTT	(3-[5,5-dimethylthyazol-2-yl-2,5-diphenyltetrazolium)
SNP	Sodium nitroprusside
BT474	Breast cancers
CHAGO-K1	Lung cancers
HEP-G2	Hepatoma cancers
KATO-3	Gastric cancers
SW620	Colon cancers

CHAPTER I
INTRODUCTION

Drug use is increasing; in 2010 cancer drugs accounted for 35 % of health spending (Cohen *et al.*, 2012). Nowadays, more people are paying attention to health care because of the toxic environment that can cause diseases, the most common being cancer (Hill, 1965). Free radicals are created as a consequence of metabolism system disorders which consist of two processes: generally reactive oxygen species (ROS), and reactive nitrogen species (RNS). These species play a role in generating oxidative stress, a deleterious process that can damage all cell structures (Pham-Huy *et al.*, 2008). The human body however has several mechanisms to counteract oxidative stress by producing antioxidants. These are either naturally produced in situ or externally supplied through foods supplements and biological systems (Mittler, 2002). The use of synthetic antioxidants in food products, allowed under strict regulation, makes these dietary protein compounds potential health hazards (Bosch-Morell *et al.*, 2002).They reported that specific amino acids of proteins can interact with free radicals if the energy of the free radical is high. The most reactive included the imidazole and aromatic functional group of amino acids (Bhatnagar *et al.*, 2011). However, the majority of the antioxidative peptides derived from food sources have low molecular weights (Sefatie *et al.*, 2013).

In recent years several studies have been undertaken on the physiological properties of bioactive peptides to identify and optimize the isolate from various organisms. These peptides are generated both *in vivo* and *in vitro* from the proteolytic hydrolysis of food proteins. The enzymatic hydrolysis of proteins is one approach used to release bioactive peptides and widely applied to improve functional and nutritional properties of protein sources (Hartmann *et al.*, 2007; Korhonen *et al.*, 2006). The biological activity of a peptide is widely recognized to be

based on amino acid composition. Peptides could be used in the formulation of functional foods and nutraceuticals to prevent damage related to oxidative stress in human disease conditions. Moreover, natural antioxidants are desirable because they can be used at higher concentrations, without the toxic side effects associated with the use of synthetic equivalents (Sies, 1997).

There are wide sources and abundant content of aquatic organism proteins, especially proteins from marine organisms where activated peptides have been gaining attention in the past few years. Various marine animal sources such as grass carp (Ren *et al.*, 2008), blue mussel (Wang *et al.*, 2013), abalone (Zhou *et al.*, 2012), scallop (Zhou *et al.*, 2012), surf clam (Suprenant *et al.*, 1984), skipjack tuna (Klompong *et al.*, 2007), and croaker (Nazeer *et al.*, 2012) have also proved to be good sources of antioxidative peptides. There has been considerable interest in the commercial culture of spotted babylon, *Babylonia areolata*, in Thailand resulting from increasing demand and the expansion of the domestic seafood market, coupled with a catastrophic decline in natural spotted babylon populations in the Gulf of Thailand. No studies have reported on the anticancer activity of spotted babylon protein derived peptides.

The objective of this study was to purify, determine, and characterize the antioxidative and in vitro antiproliferative/cytotoxic activities of the protein hydrolysates obtained from the spotted Babylon.

CHAPTER II
LITERATURE REVIEWS

2.1 Free radical

The presence of free radicals in biological materials was discovered less than 50 years ago. The sciences of free radicals in living organisms are important in biology (Droge, 2002). Free radicals are continuously produced by the body's normal use of oxygen. Oxygen is an element indispensable for life. When cells use oxygen to generate energy free radicals are produced by the mitochondria. A free radical is any chemical species capable of independent existence possessing one or more unpaired electrons an unpaired electron being one that is alone in an orbital. The simplest radical is the hydrogen atom (Sisein, 2014). Free radicals are highly reactive molecules that attack the nearest stable molecule to obtain an electron. Electrons are more stable when paired together in an orbital. Potentially, this damages critical components of cells such as proteins, lipids, carbohydrates, and DNA. Therefore, biomolecules cause structural changes and loss of properties. There are two common forms of free radicals, namely, reactive oxygen species (ROS) and reactive nitrogen species (RNS). These are present and generated by exogenous sources such as ionizing radiation including oxygen ions, which can be generated during times of environmental stress (exogenous sources can be produced from pollutants, tobacco, smoke, drugs, xenobiotics, or radiation.) It has been established that ROS and RNS can be both harmful and beneficial in biological systems depending on the environment and concentration (Brown *et al.*, 2006).

2.1.1 Reactive Oxygen Species (ROS)

ROS are a group of chemically reactive molecules derived from oxygen (O_2) They are formed as natural byproducts of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. The main members of this

group are superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$) and hydrogen peroxide (H_2O_2). The other members may include hypochlorous acid (HOCl), lipid peroxides, oxygen itself ($^1O_2$) and Ozone (O_3). alkoxyl radical ($RO^{\bullet}$) and peroxyl radical ($ROO^{\bullet}$).

2.1.2 Reactive nitrogen species (RNS)

RNS are a group of chemically reactive molecules derived from nitric oxide (NO). The main members are peroxynitrite (ONOO-), nitrogen dioxide (NO2) and S-nitrosothiols. Other members include dinitrogen trioxide (N2O3), various NO derivatives of fatty acids and NO itself, which combines with a hydrogen atom to make water and a fatty acid radical

2.2 Oxidative stress

Oxidative stress (Sisein, 2014) is defined as an imbalance between the production of free radicals and reactive metabolites so called oxidants or reactive oxygen species and their elimination by protective mechanisms, referred to as antioxidants. This imbalance leads to the damage of important biomolecules and cells, with potential impact on the whole organism. The harmful effects of ROS are balanced by the action of antioxidants, some of which are enzymes present in the body. Despite the presence of the cell's antioxidant defense system to counteract oxidative damage from ROS, oxidative damage accumulates during the life cycle and has been implicated in diseases, aging and age dependent diseases such as cardiovascular disease, cancer, neurodegenerative disorders and other chronic conditions.

2.3 Diseases caused by oxidative stress

When produced in excess, free radicals and oxidants generate a phenomenon called oxidative stress, a deleterious process that can seriously alter cell membranes and other structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA). Oxidative stress can arise when cells cannot adequately destroy the excess of free radicals formed. The body has several mechanisms to counteract these attacks by using DNA repair enzymes and antioxidants. If not regulated properly, oxidative stress can induce a variety of chronic and degenerative diseases as well as the aging process and some acute pathology (Pham-Huy *et al.*, 2008)

2.3.1 Cancer disease

The development of cancer in humans is a complex process including cellular and molecular changes mediated by diverse endogenous and exogenous stimuli. It is well established that oxidative DNA damage is responsible for cancer development. Cancer initiation and promotion are associated with chromosomal defects and oncogene activation induced by free radicals. A common form of damaged is the formation of hydroxyled bases of DNA, which is considered an important event in chemical carcinogenesis. This adduct formation interferes with normal cell growth by causing genetic mutations and altering normal gene transcription. Oxidative DNA damage also produces a multiplicity of modifications in the DNA structure including base and sugar lesions, strand breaks, DNA-protein cross-links and base-free sites. For example, tobacco smoking and chronic inflammation resulting from noninfectious diseases like asbestos are sources of oxidative DNA damage that can contribute to the development of lung cancer and other tumors. The highly significant correlation between consumption of fats and death rates from leukemia and breast, ovary, rectum cancers among elderly people may be a reflection of greater lipid peroxidation (Ozben, 2007).

2.3.2 Cardiovascular disease

Cardiovascular disease (CVD) is of multifactorial etiology associated with a variety of risk factors for its development including hypercholesterolemia, hypertension, smoking, diabetes, poor diet, stress and physical inactivity amongst others. Recently, research data has prompted a passionate debate as to whether oxidative stress is a primary or secondary cause of many cardiovascular diseases. Further in vivo and ex vivo studies have provided precious evidence supporting the role of oxidative stress in a number of CVDs such as atherosclerosis, ischemia, hypertension, cardiomyopathy, cardiac hypertrophy and congestive heart failure (Lakshmi *et al.*, 2009).

2.3.3 Neurological disease

Oxidative stress has been investigated in neurological diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), memory loss and depression. In a disease such as Alzheimer's, numerous experimental and clinical studies have demonstrated that oxidative damage plays a key role in the loss of neurons and the progression to dementia. The production of ß-amyloid, a toxic peptide often found present in the Alzheimer's patient's brain, is due to oxidative stress and plays an important role in the neurodegenerative processes (Uttara *et al.*, 2009).

2.3.4 Pulmonary disease

There is now substantial evidence that inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) are characterized by systemic and local chronic inflammation and oxidative stress. Oxidants may play a role in enhancing inflammation through the activation of different kinases and redox transcription factors such as NF-kappa B and AP-1(Rahman, 2005)

2.3.5 Rheumatoid arthritis

Rheumatoid arthritis is an autoimmune disease characterized by chronic inflammation of the joints and tissue around the joints with the infiltration of macrophages and activated T cells. The pathogenesis of this disease is due to the generation of ROS and RNS at the site of inflammation. Oxidative damage and inflammation in various rheumatic diseases have been proven to be caused by increased levels of isoprostanes and prostaglandins in serum and synovial fluid compared to controls (Hitchon *et al.*, 2004).

2.3.6 Nephropathy disease

Oxidative stress plays a role in a variety of renal diseases such as glomerulonephritis and tubulointerstitial nephritis, chronic renal failure, proteinuria, and uremia. The nephrotoxicity of certain drugs such as cyclosporine, tacrolimus (FK506), gentamycin, bleomycin, and vinblastine is mainly due to oxidative stress via lipid peroxidation (27-30). Different heavy metal (Cd, Hg, Pb, As) and transition metal (Fe, Cu, Co, Cr)-induced forms of nephropathy and carcinogenicity are strong free radical inducers in the body (Kashihara *et al.*, 2010).

2.4 Antioxidation activity

The term “antioxidant” refers to any molecule capable of stabilizing or deactivating free radicals before they attack cells. Humans have evolved highly complex antioxidant systems (enzymatic and nonenzymatic), which work synergistically, and in combination with each other to protect the cells and organ systems of the body against free radical damage. Antioxidants can be endogenous or obtained exogenously such as through part of a diet or as dietary supplements. Some dietary compounds that do not neutralize free radicals, but enhance endogenous activity may also be classified as antioxidants.

An ideal antioxidant should be readily absorbed and quench free radicals, and chelate redox metals at physiologically relevant levels. Endogenous antioxidants play a crucial role in maintaining optimal cellular functions and thus systemic health and wellbeing. However, under conditions, which promote oxidative stress, endogenous antioxidants may not be sufficient and dietary antioxidants may be required to maintain optimal cellular functions. Some antioxidants can interact with other antioxidants regenerating their original properties; this mechanism is often referred to as the antioxidant network. There is growing evidence to support a link between increased levels of ROS and disturbed activities of enzymatic and non-enzymatic antioxidants in diseases (Sisein, 2014).

2.5 Example of antioxidant

2.5.1 Natural antioxidant

2.5.1.1 Enzymatic antioxidants

2.5.1.1.1 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is one of the most effective intracellular enzymatic antioxidants and it catalyzes the conversion of superoxide anions to dioxygen and hydrogen peroxide. Superoxide dismutase exists in several isoforms, which differ in the nature of active metal center, amino acid composition, co-factors and other features. There are three forms of SOD present in humans: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD, and extra cellular-SOD. Superoxide dismutase neutralizes superoxide ions by going through successive oxidative and reductive cycles of transition metal ions at its active site. Cu, Zn-SOD has two identical subunits with a molecular weight of 32 kDa and each of the subunit contains an active site, a dinulcear metal cluster constituted by copper and zinc ions, and which specifically catalyzes the dismutation of the superoxide anion to oxygen and water. The mitochondrial Mn-SOD is a homotetramer with a molecular weight of 96 kDa and

contains one manganese atom per subunit, and it cycles from Mn (III) to Mn (II), and back to Mn (III) during the two-step dismutation of superoxide. Extra cellular superoxide dismutase contains copper and zinc, and is a tetrameric secretary glycoprotein having a high affinity for certain glycosaminoglycans such as heparin and heparin sulphate; however, its regulation in mammalian tissues occurs primarily in a manner coordinated by cytokines, rather than as a response to oxidative stress (Oberley *et al.*, 1979).

$$2O_2 + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Figure 1 Elimination of superoxide radical by superoxide dismutase

2.5.1.1.2 Catalase

This enzyme is present in the peroxisome of aerobic cells and is very efficient in promoting the conversion of hydrogen peroxide to water and molecular oxygen. Catalase has one of the highest turnover rates for all enzymes one molecule of catalase can convert approximately 6 million molecules of hydrogen peroxide to water and oxygen each minute (Ando *et al.*, 2008).

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Figure 2 Elimination of hydrogen peroxide by catalase

2.5.1.1.3 Glutathione peroxidase

Glutathione peroxidases have two forms of this enzyme, one which is selenium-dependent (GPx, EC1.11.1.19) and the other, which is selenium-independent (glutathione-Stransferase, GST, EC2.5.1.18). The differences are due to the number of subunits, catalytic mechanism, and the binding of selenium at the active center, and glutathione metabolism is one of the most important antioxidative defense mechanisms present in the cells. There are four different Se-dependent glutathione peroxidases present in humans and these are known to add two electrons to reduce peroxides by forming selenoles (Se-OH) and the antioxidant

properties of these seleno-enzymes allow them to eliminate peroxides as potential substrates for the Fenton reaction. Selenium-dependent glutathione peroxidase acts in association with tripeptide glutathione (GSH), which is present in high concentrations in cells and catalyzes the conversion of hydrogen peroxide or organic peroxide to water or alcohol while simultaneously oxidizing GSH. It also competes with catalase for hydrogen peroxide as a substrate and is the major source of protection against low levels of oxidative stress. However, the most important H_2O_2 removing enzymes in human cells are glutathione peroxidises (GSHPX), enzymes that require selenium (has selenocysteine at the active site) for their action. GSHPX enzymes remove H_2O_2 by using it to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase, a FADcontaining enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power (Battin *et al.*, 2009).

$$2GSH + ROOH \xrightarrow{GPX} GSSG + ROH + H_2O$$

Figure 3 Elimination of lipid peroxide by glutathione peroxidase

2.5.1.2 Non-enzymatic antioxidants

2.5.1.2.1 Vitamin C (L–Ascorbic acid)

Vitamin C (L–Ascorbic acid) has a 6-carbon lactone ring structure with 2, 3 enediol moiety. The antioxidant activity of ascorbic acid comes from 2, 3 enediol. Vitamin C (L–Ascorbic acid) first changes to semi-dehydroascorbic acid through donating 1 hydrogen atom and electron, and then L–dehydroascorbic acid by donating a 2nd hydrogen atom and electron. Both L–ascorbic acid and L–dehydroascorbic acid retain the vitamin C activity. Vitamin C (L–Ascorbic acid) is highly susceptible to oxidation in the presence of metal ions such as Cu2+ and Fe3+. Oxidation of ascorbic acid is also influenced by heat, light exposure, pH, oxygen concentration, and water activity. The antioxidant mechanisms of ascorbic acid are based on the hydrogen atom donation to lipid radicals, quenching of singlet oxygen,

and removal of molecular oxygen. Scavenging aqueous radicals and regeneration of alpha–tocopherol from the ocopheroxyl radical species are also well known antioxidant mechanisms of ascorbic acid. Vitamin C (L–Ascorbic acid) is an excellent electron donor because of the low standard 1-electron reduction potential (282 mV), the generation of relatively stable semi-dehydroascorbic acid, and the easy conversion of dehydroascorbic acid to ascorbic acid (Bendich *et al.*, 1986).

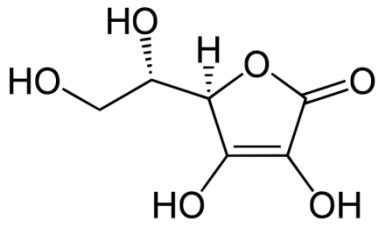


Figure 4 Vitamin C (L–Ascorbic acid of structure)

2.5.1.2.2 Vitamin E (Alpha-tocopherol)

This is a fat-soluble vitamin that exists in eight different forms. In humans, alpha-tocopherol is the most active form, and is the major powerful membrane bound antioxidant employed by the cell. The main function of vitamin E is to protect against lipid peroxidation, and there is also evidence to suggest that alpha-tocopherol and ascorbic acid function together in a cyclic-type of process. During the antioxidant reaction, alpha-tocopherol is converted to alpha-tocopherol radical by the donation of labile hydrogen to a lipid or lipid peroxyl radical. The alpha-tocopherol radical can therefore be reduced to the original alpha-tocopherol form by ascorbic acid. Antioxidant mechanisms of tocopherols include the transfer of a hydrogen atom at the 6–hydroxyl group on the chroman ring, and scavenging of singlet oxygen and other reactive species. Tocopherols are regenerated in the presence of ascorbic acids. The phytyl chain in tocopherols can fit in the membrane bilayer while the active chroman ring is closely positioned to the surface. This unique

structure enables tocopherols to act as effective antioxidants and to be regenerated through reaction with other antioxidants such as ascorbic acid (Chow, 1991).

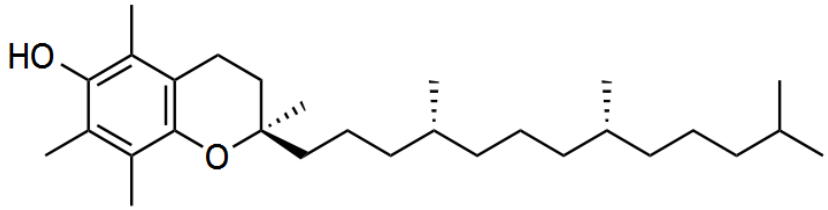


Figure 5 Vitamin E (Alpha-tocopherol of structure)

2.5.1.2.3 Carotenoids

Carotenoids are a group of tetraterpenoids. The basic carotenoid structural backbone consists of isoprenoid units formed either by head-to-tail or by tail-to-tail biosynthesis. There are primarily two classes of carotenoids: carotenes and xanthophylls. Carotenes are hydrocarbon carotenoids and xanthophylls contain oxygen in the form of hydroxyl, methoxyl, carboxyl, keto, or epoxy groups. Lycopene and beta–carotenes are typical carotenes whereas lutein in green leaves and zeaxanthin in corn are typical xanthophylls. The structures of carotenoids are acyclic, monocyclic, or bicyclic. For example, lycopene is acyclic, grammar–carotene is monocyclic, and alpha– and beta–carotenes are bicyclic carotenoids. Double bonds in carotenoids are conjugated and transformations of carotenoids are found in plant tissues. Epidemiological studies have revealed that a diet rich in carotenoids correlates with a lower risk of age-related diseases. Carotenoids contain conjugated double bonds and their antioxidant activity arises due to their ability to delocalize unpaired electrons. This is also responsible for the ability of carotenoids to physically quench singlet oxygen without degradation and for the chemical reactivity of carotenoids with free radicals. The efficacy of carotenoids for physical quenching is related to the number of conjugated double bonds present in the molecule (Edge *et al.*, 1997).

2.5.1.2.4 Curcumin

Curcumin is a yellow pigment, the major constituent of turmeric. It is a diferuloylmethane with an unsaturated beta-diketone, and phenolic groups. It exhibits a variety of pharmacological properties such as anti-inflammatory, anti-carcinogenic, anti-microbial, neuro-protective, cardio-protective, thrombo-suppressive and anti-diabetic actions. The compound is considered a potent anti-cancer agent and is currently being evaluated in different stages of clinical trials against a variety of cancers. Curcumin is also a potent antioxidant. Studies from our laboratory as well as others have shown it to be an excellent scavenger of ROS and RNS, whose production is implicated in the induction of oxidative stress. Further, studies on the mechanistic aspects of antioxidant activity revealed that phenolic hydroxyl groups of curcumin play a significant role in a diverse range of antioxidant activities (Kunwar *et al.*, 2011).

2.5.1.3 Bioactive peptides

Bioactive peptides have been known to be part of the human diet for several years. With the appearance of chromatographic methods the number of studies on bioactive peptides from animal and plant sources mounted. As the findings of these studies have shown, peptides exert regulatory functions besides their nutritional roles. Bioactive peptide can be used as a basic compound of functional foods, nutraceuticals and dietary supplements. Moreover, they have the capability to be used as constituents of pharmaceuticals. Some bioactive peptides have already been commercialized and are commercially available in many countries. Moreover, there is a need to develop modern techniques to enrich active peptide in food proteins and to facilitate the production of these peptides in huge amounts for the market. There are few clinical human studies on the health benefits of bioactive peptides; thus,

further study should be conducted to explain the physiological importance of these peptides in humans (Sarmadi *et al.*, 2010).

2.5.2 Synthesis antioxidant

The use of synthetic compounds possessing antioxidant activity for the preservation of cosmetic, pharmaceutical and food products has been a common practice.

2.5.2.1 Butylated hydroxytoluene (BHT)

BHT, also known as butylhydroxytoluene, is a lipophilic organic compound, chemically a derivative of phenol, which is useful for its antioxidant properties. European and U.S. regulations allow small amounts to be used as food additives. In addition, BHT is widely used to prevent oxidation in fluids (for example, fuel and oil) and other materials where free radicals must be controlled. The species behaves as a synthetic analog of vitamin E, primarily acting as a terminating agent that suppresses autoxidation, a process whereby unsaturated (usually) organic compounds are attacked by atmospheric oxygen. BHT stops this autocatalytic reaction by converting peroxy radicals to hydroperoxides. It affects this function by donating a hydrogen atom (Lambert *et al.*, 1996).

$$RO_2^{\cdot} + ArOH \longrightarrow ROOH + ArO^{\cdot}$$

$$RO_2^{\cdot} + ArO^{\cdot} \longrightarrow \text{Non-radical products}$$

Where R is alkyl or aryl, and where ArOH is BHT or related phenolic antioxidants, each BHT consumes two peroxy radicals.

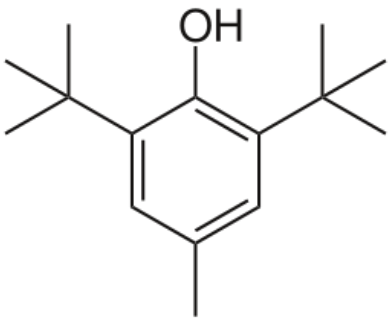


Figure 6 Butylated hydroxytoluene (BHT)

2.5.2.2 Butylated hydroxyl anisole (BHA)

Synthetic phenolic antioxidants butylated hydroxyl anisole ($C_{11}H_{16}O_2$) (Botterweck *et al.*, 2000) is the most commonly used of antioxidants in food products. This is an antioxidant consisting of a mixture of two isomeric organic compounds – 2-tert-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole. The conjugated aromatic ring of BHA is able to delocalize free radicals to inhibit oxidation reduction. BHA also known as BHA is a food antioxidant available dissolved in propylene glycol. Butylated hydroxyanisole comes as a white crystalline powder or a yellowish-white waxy solid. It is used in a wide range of cosmetics, foods, and pharmaceuticals. When used in food products, it delays the oxidative rancidity of fats and oils, and prevents the loss of activity of oil-soluble vitamins. It may be found in pharmaceutical gels, creams and liquid or gelatin capsules, tablets and in other pharmaceutical dosage forms. Animal studies have shown tumors in the fore-stomachs of rats and mice at much higher levels of butylated hydroxyanisole comsumption by humans. Overall, the evidence does not support the occurrence of tumors when butylated hydroxyanisole is ingested at much lower levels.

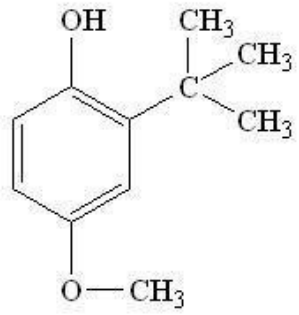


Figure 7 Butylated hydroxyl anisole (BHA)

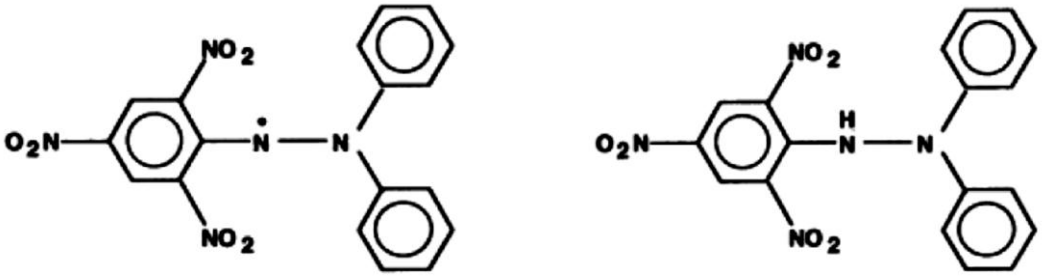
2.6. Antioxidation assays

To evaluate the antioxidative activity of specific compounds or extracts, the latter are allowed to react with a stable radical as follows:

2.6.1 DPPH radical scavenging

The molecule of DPPH is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimers, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet color, characterized by an absorption band in ethanol solution center at about 514 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, this then gives rise to the reduced form with the loss of the violet color (although there would be expected to be a residual pale yellow color from the picryl group still present). With $Z^{\bullet}$ representing the DPPH radical and AH the donor molecule, the primary reaction is $Z^{\bullet} + AH = ZH + A^{\bullet}$. Where ZH is the reduced form and $A^{\bullet}$ is the free radical produced in this first step, this latter radical will then undergo further reactions which control the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorized) by one molecule of the reductant. The reaction ($Z^{\bullet} + AH = ZH + A^{\bullet}$) is therefore intended to provide the link with the reactions taking

place in an oxidizing system, such as the autoxidation of a lipid or other unsaturated substance; the DPPH molecule $Z^{\bullet}$ is thus intended to represent the free radicals formed in the system whose activity is to be suppressed by the substance AH (Molyneux, 2004).



1: Diphenylpicrylhydrazyl (free radical)

2: Diphenylpicrylhydrazine (nonradical)

Figure 8 DPPH- free radical and reduced form

2.6.2 ABTS radical scavenging

Generation of the ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline 6-sulfonic acid)] radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of the solutions of pure substances, aqueous mixtures and beverages. The original $ABTS^{\bullet+}$ assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation, in the presence or absence of antioxidants. This has been criticized on the basis that the faster reacting antioxidants might also contribute to the reduction of the ferryl myoglobin radical. A more appropriate format for the assay is a decolonization technique in which the radical is generated directly in a stable form prior to reaction with putative antioxidants. The improved technique for the generation of $ABTS^{\bullet+}$ described here involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between

ABTS and potassium persulfate. This has absorption maxima at wavelengths 645 nm, 734 nm and 815 nm, as reported previously, as well as the more commonly used maximum at 415 nm. Addition of antioxidants to the pre-formed radical cation reduces ABTS, to an extent and on a time-scale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction. Thus, the extent of decolonization as percentage inhibition of the $ABTS^{\bullet+}$ radical cation is determined as a function of concentration and time and calculated relative to the reactivity of a standard, under the same conditions. The method is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts (Moreno, 2002).

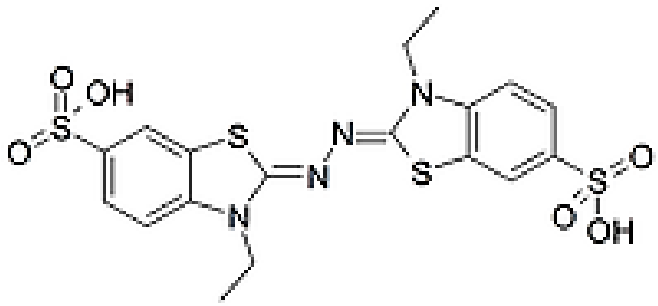


Figure 9 ABTS radical

2.6.3 Nitrite oxide radical scavenging

One method for the indirect determination of NO involves the spectrophotometric measurement of its stable decomposition products NO_3^- and NO_2^-. This method requires that NO_3^- first be reduced to NO_2^- which is then in turn determined by the Griess reaction as shown in Figure 10. Briefly, the Griess reaction is a two-step diazotization reaction in which the NO derived nitrosating agent, dinitrogen trioxide (N_2O_3) generated from the acid-catalyzed formation of nitrous acid from nitrite (or autoxidation of NO) reacts with sulfanilamide to produce a diazonium ion which is then coupled to N-(1-napthyl) ethylenediamine to form a chromophoricazo product that absorbs strongly at 540 nm (Bryan *et al.*, 2007).

The nitrosating agent dinitrogen trioxide (N_2O_3) generated from acidified nitrite (or from the autoxidation of NO) reacts with sulfanilamide to yield a diazonium derivative. This reactive intermediate will interact with N-1-naphthylethelene diamine to yield a colored diazo product that absorbs strongly at 540 nm.

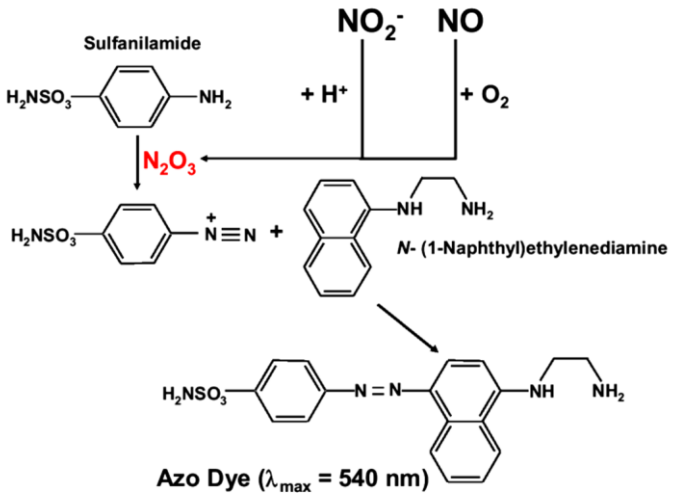


Figure 10 the Griess Reaction

2.7 Anticancer peptides

Anticancer peptides were synthetic cationic peptides or positively charge amino acids such as arginine (Arg), lysine (Lys), histidine (His) and hydrophobic group (Shai, 1999) have been reported to show anticancer activity with characteristics including the ability to kill target cells rapidly, the broad spectrum of activity, and the specificity for cancer cells (Wang *et al.*, 2009). Of all the proposed mechanisms, 2 general effects of anticancer peptides against cancer cells were suggested: cytoplasmic membrane disruption *via* micellization or pore formation, and induction of apoptosis. In addition to these effects, some anticancer peptides have been reported to display anticancer activity via different mechanisms. This peptide targets cancer cells through the interaction with cell surface gangliosides and induces mitochondria-dependent apoptosis (Lee *et al.*, 2008). The peptide group exhibits selective cytotoxicity attributed to its electrostatic interaction with surface-exposed

phosphatidylserine in cancer cells and activates the lysosomal-mitochondrial death pathway and involves autophagy-like cell death (Maher *et al.*, 2006). Compared with the traditional cancer treatments such as chemotherapy or radioactive treatment, peptides with high specificity against cancer cells may present the way of killing cancer cells while protecting normal cells and helping patients to recover rapidly.

Table 1 Anticancer of Protein hydrolysate peptides.

Source of protein hydrolysates	Preparation	Anticancer peptide	Peptide and amino acid	Reference
Germinated Soybean	pepsin and pancreatin	cervical uterine cancer	MW >10 kDa	Mora-Escobedo *et al.* (2009)
Shrimp shell	Cryotin	colon cancer	MW >10 kDa	Kannan *et al.* (2011)
Blue mussel	Pepsin	prostate cancer, lung cancer and breast cancer	Ala-Phe-Asn-Ile-His-Asn-Arg-Asn-Leu-Leu	Kim *et al.* (2012)

2.8 Proteolytic enzyme

Protein hydrolysate is a fascinating one. Which, it is a natural substance and Bioactive Protein hydrolysate a product of protein degradation by cutting lines polypeptide. These peptides bond short chain peptide which consists of amino acids and have important biological activity. Enzymatic hydrolysis process used to digest proteins at the peptide bond. The peptides bond is split among carboxyl acid and the amino acid. by paying attention proteolytic enzyme capable of degrading proteins divided into three groups such as enzymes derived from animals such as

pepsin pancreatin, alpha-chymotrypsin, enzymes derived from plants such as papain, enzymes from microorganisms such as alcalase, neutrase (Neurath *et al.*, 1976).

2.9 Protein hydrolysates

Protein hydrolysate (Silvestre, 1997) is a mixture consisting of peptide or short chain protein obtained from the hydrolysis of long chain protein via acid or base treatment or enzyme. In this thesis, enzymatic hydrolysis was chosen to select the hydrolyse protein of human and plants. Several protein hydrolysates are reported to possess antioxidant activity as described above in section 2.5.1.3. Pepsin and pancreatin were used as enzymes in the hydrolysis process because both of them are actually involved in the human digestive system with pepsin located in the stomach and pancreatin secreted in the small intestine. The protein hydrolysate gained from these enzymes provides properties that are similar to the protein hydrolysate obtained through the digestion in humans in real life (Hur *et al.*, 2011). Papain was used as enzymes in the hydrolysis process because papain was plants digestive system with papain Isolated from Papaya (*Carica papaya*) Latex. Papain enzyme belongs to the papain superfamily, as a proteolytic enzyme, papain is of crucial importance in many vital biological processes in all living organisms (Mamboya, 2012).

Pepsin (E.C. 3.4.23.1) is the enzyme in gastric juice that can digest proteins. It is first secreted as pepsinogen which is a pro-enzyme and is therefore still unable to functionalize. After mixing with hydrochloric acid, pepsinogen turns itself into an active enzyme pepsin. The optimal pH is 1.5-2.5 for pepsin which, proteolytic enzyme for the proteinase group cut endropeptidase type rang Phenylalanine (Phe) and Leucine (Leu), as mentioned, is located in the stomach Therefore, pepsin is no longer active in the small intestine with its pH over 1.5-2.5 at temperature 37-42°C. Pepsin can only digest protein into peptide or shorter proteins, but not into a single amino acid.

Pancreatin (E.C. 232-468-9) is a mixture of enzymes released from the pancreas including amylase, lipase, and trypsin. Trypsin is a protein hydrolysable enzyme which can digest protein after it has been digested by pepsin in the stomach

which, proteolytic enzyme for the proteinase group cut Endropeptidase type rang Leucine (Leu) and Arginine (Arg). The optimal pH is 7-8 at temperature 37-42°C (Abdel-Aal, 2008).

Papain is a plant proteolytic enzyme for the cysteine proteinase family cysteine protease enzyme cut Endropeptidase type rang Leucine (Leu) and Glycine (Gly). The enzyme is able to break down organic molecules made of amino acids, known as polypeptides and thus plays a crucial role in diverse biological processes. The optimal pH is 6-7at temperature 60-65°C.

Table 2 Source of protein hydrolysates

Source of protein hydrolysates	Preparation	Antioxidant activity	Peptide and amino acid	Reference
Abalone viscera	Alkali protease, papain and neutral	DPPH, Reducing powder and hydroxyl radical	small-size peptide (blow 200 Da)	Zhou *et al.* (2012)
Blue mussel	Pepsin, papain, neutrase and alcalase	DPPH, Superoxide anion and hydroxyl radical	Tyr-Pro-Pro-Ala-Lys	Wang *et al.* (2013)
Croker muscle	Pepsin, trypsin and alpha-chymotrypsin	DPPH, hydroxyl radical and lipid proxidation	Lys-Thr-Phe-Cys-Gly-Arg-His	Nazeer *et al.* (2012)
Grass carp	Papain, pancreantin, neutrase and alcalase	Hydroxyl radical and lipid peroxidation	Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val	Ren *et al.* (2008)

Continue

Source of protein hydrolysates	Preparation	Antioxidant activity	Peptide and amino acid	Reference
Scallop	Neutral protease and papain	DPPH, hydroxyl radical and reducing powder and ferrousion chelating capacity	small-size peptide (blow 500 Da)	Zhou *et al.* (2012)
Yellow stripe trevally	Alcalase and flavourzyme	DPPH, Reducing powder and Metal-chelating	small-size peptide	Klompong *et al.* (2007)
Royal jelly	Protease N	Hydroxyl radical, metal-chelating, superoxide-anion	Lys-Tyr, Arg-Tyr, and Tyr-Tyr	Guo *et al.* (2009)
Sphyrna lewini muscle	Papain	DPPH	Trp-Asp-Arg and Pro-Tyr-Phe-Asn-Lys	Wang *et al.* (2012)

2.10 Spotted Babylon snail

The spotted Babylon snail (*Babylonia areolata*) is a marine gastropod of interest as a new source of protein for human consumption from potential production in aquaculture. It can be found in the coastal waters of the Gulf of Thailand and Andaman Sea off Thailand. It has a very high nutritional value compared with sea shells and other economic marine species such as grouper, mackerel, mussels, oysters, abalone, shrimp, crab and octopus (Table 3). It has significant economic importance and there is high demand in markets throughout Asia. The spotted babylon has higher protein content than mussels, oysters and abalone. Also, the protein content is similar to that of fish and shrimps containing 20.01% protein and 2.39% lipid. Lastly, it contains 17 types of amino acids as well as 20 kinds of fatty acids. Together with its biological activity (bioactive protein hydrolysate), the spotted babylon is a new and promising source of protein hydrolyzate (Chaitanawisuti *et al.*, 2011)

Table 3 Nutritional value of other economic marine species.

Animal	Nutritional value (100 g)				
	Protein	Total fat	Total Ash	Carbohydrate	Water
Spotted Babylon	20.01	2.3	2.38	3.33	73.87
Blue mussel	11.9	2.24	1.59	3.69	80.58
Oysters	9.45	2.3	1.23	4.95	82.06
Abalone	17.1	0.76	1.57	6.01	74.56
Octopus	16.24	0.7	1.68	0.82	80.56
Mackerel	19.29	6.3	1.27	0	71.67
Crab	18.06	1.08	1.81	0.04	79.02
Shrimp	20.31	1.73	1.2	0.9	75.86

Source: Seafood Health Facts (2008)

2.10.1 Taxonomy of spotted Babylon snails (*Babylonia areolata*, Link, 1807)

Common name: Spotted Babylon snails

Thai name: หอยหวาน

Phylum: Mollusca

Class: Gastropoda

Order: Neogastropoda

Family: Buccinidae

Genus: *Babylonia*

It has been reported that its biological substance comprises mostly small organic molecules, with very few reports of protein hydrolyzate with biological activity. This is attractive, particularly in finding the active ingredient in the protein hydrolyzate, especially protein hydrolyzate bioactive in the treatment of cancer and for health. Significant increases in the current. The data will be used in medical, pharmaceutical, and industrial applications or further research. Production as an ingredient in medicines, cosmetics, and food, spotted babylon can be used for commercial benefit.

CHAPTER III

EXPERIMENTS

3.1 Raw material

Spotted babylon were captured from a farm at Sichang Marine Science Research and Training Station, Chulalongkorn University, located on Sichang Island, Chonburi Province, Thailand. The samples were taken immediately to the laboratory and kept refrigerated until required.

3.2 Chemical and Biological material.

2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), bovine serum albumin (BSA), 1, 1-Diphenly-2- picrylhydrazyl (DPPH), MTT (3-[5,5-dimethylthyazol-2-yl-2,5-diphenyltetrazolium), napthylethylenediamine dihydrochloride (NED), pancreatin from porcine pancreas, pepsin from porcine gastric mucosa, potassium persulfate, and sodium nitroprusside (SNP) were purchased from Sigma, (USA). Biomax® 5 and Biomax® 10 were purchased from Amersham Biosciences, (Sweden). All other unlabeled chemicals and reagents were of analytical grade. The five human tumor cell lines, BT474 (breast), CHAGO-K1 (lung), HEP-G2 (hepatoma), KATO-3 (gastric), and SW620 (colon), were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand.

3.3 Preparation of Spotted Babylon snails.

The spotted babylon snail was first anaesthetized with ice. The muscle was separated and blended using a mixer. The homogenate was then defatted following the method described by Klompong *et al.* (2007), by isopropanol at a ratio of 1:4 (w/v), at room temperature for 1 h. The supernatant was then removed by vacuum filtration and dried at 60 °C (overnight). The resultant powder was thoroughly blended using on electric generator and the dried homogenate was stored at room temperature in desiccators.

3.4 Total amino acid analysis

The method described by Chirinang *et al.* (2009) was followed. Five mg of spotted babylon snail powder was hydrolyzed into 5 ml of 6N HCl and then placed in a heating block at 110 ℃ for 22 h. The internal standard was 10 ml of 2.5 mM L- alpha -amino-n-butyric acid in 0.1 M HCl. The solution was diluted with 250 ml deionized water (DI water), and then filtered through 2.0 µm microfiltration membrane. Then 6M AccQ-Fluor derivative buffer and AccQ-Fluor reagent were added to the derivation and it was heated at 55 ℃ for 10 min in a heating block. The heating converted the minor side product of tyrosine to a major monoderivative compound. Total amino acid content was determined by high performance liquid chromatography using Waters 2695 Alliance Separation Module (Milford, MA, US). Analyses were separated on a Hypersil GOLD column (Waters), eluted isocretically with a sodium acetate buffer pH 4.90 and 60 % acetonitrile, delivered at a flow rate of 0.3 ml/min. The injection was 5 µl. The total run time was 15 min.

3.5 Preparation of Spotted Babylon snail's protein hydrolysates with Enzyme

3.5.1 Pepsin and pancreatin.

The spotted babylon powders were used as a substrate for production of the protein hydrolysate following the method of Sefatie *et al.* (2013). Each crude protein preparation was performed for eight concentrations (0.5, 0.25, 0.1, 0.075, 0.05, 0.025, 0.0125, and 0.00625 g/ml), mixed with pepsin until the final substrate/enzyme (g/g) concentration ratio was 20:1 (ml/g) and then adjusted to pH 2.5 by adding 1M HCl. The hydrolysis was carried out for 180 min at 37 ℃ with shaking (180 rpm), and then inactivated by adding 1M NaOH to pH 7.5. Next, pancreatin was added to a 20:1 (ml /g) substrate to enzyme ratio, and shaken (180 rpm) for 180 min at 37 ℃. The hydrolysis (enzyme reaction) was stopped by heating at 90 ℃ for 20 min. Hydrolysates were clarified by centrifugation 15,000×g, 30 min, 4 ℃, and then

freeze-dried and stored at -20 °C until use. These two proteases were chosen to crudely mimic those in the human gastro-intestinal tract.

3.5.2 Papain.

The spotted babylon powders were used as a substrate for production of the protein hydrolysate following the method of You *et al.* (2011). Each crude protein preparation at eight concentrations (0.5, 0.25, 0.1, 0.075, 0.05, 0.025, 0.0125, and 0.00625 mg/ml) was mixed with papain until the final substrate/enzyme (ml/g) concentration ratio was 20:1 (ml/g) and adjusted to pH 6.5 with 1N KOH. The hydrolysis was carried out for 180 min at 60 °C with shaking (180 rpm). The hydrolysis (enzyme reaction) was stopped by heating at 90 °C for 20 min. Hydrolysates were clarified by centrifugation 15,000×g, 30 min, 4 °C, and then freeze-dried and stored at -20 °C until use. The proteases were chosen to crudely mimic those in the plant.

3.6 Molecular weight cut off by ultrafiltration

The spotted babylon protein hydrolysates were dissolved in distilled water and fractionated through ultrafiltration membranes using a bioreactor system (Amersham Biosciences, Sweden). Spotted babylon peptide solution was pumped through a range of nominal molecular weight cut-off (MWCO) membranes of 10, 5, and 3 kDa, respectively (Park *et al.*, 2001). Five fractions were collected from the membrane filtration, including retentate from the 10 kDa membrane (MW >10 kDa), retentate from the 5 kDa membrane (MW 5 – 10 kDa), retentate from the 3 kDa membrane (MW 3 – 5 kDa), permeate from the 5 kDa membrane (MW < 5 kDa), and permeate from the 3 kDa membrane (MW < 3kDa).

3.7 Antioxidation activity

3.7.1 DPPH radical scavenging activity

The stable DPPH was used for the determination of free radical-scavenging activity of the spotted babylon protein hydrolysates. The assay was carried out following the method of Wu *et al.* (2003) with slight modifications. The sample

solution with different concentrations was mixed with DI water and 0.1 mM DPPH in methanol solution at a ratio of 1:4. After mixing, the fluid was kept in the dark at room temperature for 30 min. The absorbance at 517 nm was recorded. L-ascorbic acid (Vitamin C) was used as the standard control. The radical scavenging activity percentage was expressed as:

$$\frac{\left((A_c\text{-}A_{cb})\text{-}(A_s\text{-}A_{sb})\times 100\right)}{(A_c\text{-}A_{cb})}$$

Where Ac is the absorbance of DI water and 0.1 mM DPPH-Methanol, Acb is the absorbance of DI water and methanol, As is the absorbance of sample, and DPPH-Methanol and Asb is the absorbance of sample and methanol. The IC_{50} values gave the concentration of sample required to scavenge 50 % of the free radical.

3.7.2 ABTS scavenging activity

The ABTS free radical assay was carried out following the method of Wiriyaphan *et al.* (2012), with slight modifications. ABTS solution (7 mM) was mixed with 2.45 mM potassium persulfate in the ratio 1:1, and the solution was kept in the dark at room temperature for 12-16 h. Different concentrations of sample were mixed with DI water. The working solution of ABTS was diluted with DI water. The absorbance at 734 nm was recorded. BHT (butylated hydroxytoluene) 500µg/ml was used as the standard control. The radical scavenging activity percentage and IC_{50} values were calculated.

3.7.3 Nitric oxide scavenging activity

Nitric oxide was used for the determination of ion scavenging activity of the spotted babylon protein hydrolysates. The assay was performed according to the method of Jayan *et al.* (1997), with slight modifications. The Griess-Ilosvay reagent, composed of sodium nitroprusside (10 mM) phosphate buffer (pH 7.4), was mixed with different concentrations of sample. The mixture was then mixed with DI water at a ratio of 1:1 and incubated at room temperature for 150 min. The solution was

then added to 0.33 % sulfanilamide in 20 % acetic acid and incubated for 5 min. Then 0.1 % NED was added and incubated for 30 min at room temperature. Curcumin (500 µg/ml) was used as the standard control. The percentage of radical scavenging activity and IC_{50} values were calculated.

3.8 Protein content determination

The protein content was determined by Bradford's procedure (Dan *et al.*, 2010). Bovine serum albumin (BSA) was used as the standard with four different concentrations between 5 to 20µg/ml to construct the calibration curve.

3.9 Cytotoxicity assay of cancer cell line by MTT assays

The cytotoxicity assay for the in vitro anti-proliferative activity of different human malignant cell lines including BT474 (breast), HEP-G2 (hepatoma), CHAGO-K1 (lung), SW620 (colon), and KATO-3 (gastric) was carried out in tissue culture (Jang *et al.*, 2008). Cells were kept in complete media, composed of RPMI-1640 along with 2.0 mM L-glutamine and 5 % (v/v) fetal calf serum at 37 ℃ under 5 % (v/v) CO_2 condition. Cells were aspirated, trypsinized, and finally washed, prior to being seeded at a density of 5×10^3 cells/µl by immersion in 200 µl of complete media in a 96-well plate and cultured for 1 day. Dilutions of the sample were then added into each well (0-25 µg/ml in 200 µl of complete media), and incubated for 3 days. Then 10 µl of 3-[5,5-dimethyazol-2-yl-2,5-diphenyltetrazolium] bromide solution (5 mg/ml) (MTT) was poured into each well and incubated for 4 h prior to aspiration of the media, and then washed with RPMI-1640 to remove any media still left in the solution. One hundred and fifty µl of DMSO was then added into each well and left for 30 min. The cell remains and its solution were then aspirated, and the absorbance determined at 540 nm by microliter reader.

3.10 Apoptosis of Human lung cell line

The apoptosis program was Annexin V binding to phosphatidylserine exposed on the external surface of the plasma membrane during the early phase, propidium iodide (PI) was late apoptosis and became accessible DNA within the nucleus only (Su *et al.*, 2010). Therefore, Annexin V and PI were used together to distinguish early and late apoptosis. The lung cancer cell CHAGO-K1 was prepared in RPMI-1640 complete media in cell culture flasks at a density of 2×10^5 cells / flask / 5ml. These were incubated overnight at 37 ℃ in 5 % CO_2. The sample with IC_{20} values was added into culture flasks at a density of 2×10^5 cells / flask / 5ml and incubated for 8 h. To identify the apoptotic, the assay was carried out following the method of Murray *et al.* (2004) with slight modifications, using the FITC Annexin V Apoptosis Detection Kit with PI (BioLegend Inc., San Diego, CA, USA). The treated cells were harvested by trypsinization and washed twice with cold 20 mM phosphate-buffered saline (PBS) with 1 % fetal calf serum (FCS). Resuspended cells in 100 µl Annexin V binding buffer and cells suspension 100 µl were then transferred into a 1.5 ml micro centrifuge tube and 2.5 µl of FITC Annexin V and 5 µl of propidium iodide solution (PI) were added to each tube. The tubes were incubated for 15 min at room temperature in the dark and then 200 µl of Annexin V binding buffer was added into each tube and cells. Finally, the program was set for analysis by flow cytometry (FlowJo. Co,).

3.11 Purification of peptides by RP-HPLC

The separated < 3 kDa fraction and sample injection volume of 30 µl was analyzed by RP-HPLC on C18 column (4.6 mm × 250 mm) using a linear gradient of acetonitrile, containing 0.1 % TFA of ratios 0:100, 12:88, 25:75, 45:55, and 35:65 at a flow rate of 0.7 ml/min. The elution solution was detected at 280 nm and the peptide fraction collected every 10 min. 3.12 Identification antioxidation of peptides

3.12 Identification of peptides mass spectrometry

The molecular weight and amino acid sequence of active protein hydrolysates were identified using a direct infusion LC–MS/MS system, consisting of a liquid chromatography part (Dionex Ultimate 3000, Thermo Scientific), in combination with an electrospray ionization (ESI)/quadrupole ion trap mass spectrometer (Model Amazon SL, Bruker, Germany). The LC separation was performed on a reversed phase column (Hypersil GOLD 50 mm × 0.5 mm, 5 μm C_{18}), protected by a guard column (Hypersil GOLD 30 mm × 0.5 mm, 5 μm C_{18}), eluted at a flow rate of 100 μl/min under gradient conditions of 5 to 80 %B over 50 min. Mobile phase A consisted of water/formic acid (99.9:0.1, v/v), and B consisted of acetonitrile (100, v). Mass spectral data from 300 to 1500 m/z were collected in the positive ionization mode. All data were processed using the MASCOT database.

3.13. Statistical analysis

All investigations were performed in triplicate. The results were shown as the mean±standard deviation. GraphPad Prism (Version 6.0, GraphPad Software Inc., La Jolla, CA, USA) for Windows was used to calculate IC_{50} values. Statistic program (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL, USA) of significant difference were performed by One-Way-ANOVA and evaluated by Duncan's multiple range test with $p < .05$ accepted as significant.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Amino acid composition of spotted babylon snails

The amino acid composition of spotted babylon powders was analyzed by the acid hydrolysis method and HPLC Column (Table 4). These were in agreement with the results of the protein content. The high results for glutamic acid (Glu) and Aspartic acid (Asp) showed a very positive effect on the nervous system and assisted exercise. These were the two highest amino acid contents; they are essential and major amino acids (Yang *et al.*, 2011). There are several important amino acids, such as hydrophobic group amino acids at the N-terminus end (Leucine, Valine, Cystine, Methionine, and Proline) (Mohamed *et al.*, 2012). The imidazole functional group of histidine is aromatic and contains four two double bond forms and two nitrogen lone pair forms (Chen *et al.*, 1996). The aromatic group of amino acids (Tyrosine, Tryptophan, and Phenylalanine) exhibit antioxidant activity since several amino acids can donate protons to electron-deficient free radicals (Stadtman *et al.*, 2003). Consequently, protein hydrolysate from spotted babylon would be highly valued for biological activity and food nutrition as the amino acid composition from spotted babylon snail powder (SBP) included many important amino acids (Chaitanawisuti *et al.*, 2011). Compared with amino acid of blue mussel have 17 amino acids (Jung *et al.*, 2005) which coincided to amino acid of spotted babylon snails, These were a creature that probability new source of protein hydrolyzate. To identified bioactive peptides.

Table 4 Total amino acid composition of spotted babylon snail powders

Analyses Amino acid Profile	Result (%)
Alanine	4.36
Arginine	5.85
Glycine	4.3
Aspartic Acid	7.09
Valine*	3.03
Cystine*	0.64
Glutamic Acid	11.7
Leucine*	5.27
Isoleucine	2.36
Histidine*	1.2
Threonine	3.15
Proline*	3.12
Lysine	4.42
Methionine*	1.65
Serine	3.12
Phenylalanine*	2.43
Tyrosine*	2.37
Tryptophan*	0.4
Hydroxyproline	Not Detected
Hydroxylysine	Not Detected
Antioxidant amino acid*	20.11

*showed different values of amino acid inhibit antioxidant activity from SBP

4.2 Screening for concentrated spotted babylon by enzymatic hydrolysis inhibited antioxidant activity

Free radical scavenging activity has been considered as one of major reasons for many cancer diseases in humans. The cause of these free radicals is from a metabolism system depressive disorder which might shed excess free radicals giving rise to oxidative stress. Thus antioxidant activity is an important factor for consumption into the body. The spotted babylon were chosen for investigation as they have high nutritious value compared to the marine animal economy, containing up to 18 species of essential amino acids. The spotted babylon was interesting as a protein hydrolysate by enzymatic hydrolysis.

Protein hydrolysate of spotted babylon was prepared using pepsin and pancreatin (pancreatin included trypsin and chymotrypsin), obtained from degradation in the gastrointestinal tract of humans (Sefatie *et al.*, 2013). Papain was obtained from the decomposition process of the plant (You *et al.*, 2011). Both enzymes are in the proteinase group. Therefore, the protein hydrolysate of spotted babylon, prepared from pepsin-pancreatin and papain was most likely to possess the different peptide size and amino acid sequences to determine their capacity in antioxidant activity (Mundi *et al.*, 2014).

The antioxidant activity of the crude protein, hydrolyzed by enzymatic hydrolysis under optimal conditions (section 3.5) was measured by DPPH (section 3.7.1). The results were reported as IC_{50} values (the concentration of sample that can inhibit 50 % of antioxidant activity), calculated from the regression equation derived from the percentage inhibition versus the concentration of sample. The IC_{50} value was compared with positive standards such as L-ascorbic acid (Vitamin C), butylated hydroxytoluene (BHT), and natural antioxidant, used as a reference for comparing the IC_{50} values of samples, because these are already known antioxidants used widely in many studies.

The screening results for concentrated spotted babylon by enzymatic hydrolysis are shown in Figure 11. The concentration of 0.075 g/ml for pepsin and pancreatin compared with all concentrations of spotted babylon digested by pepsin and pancreatin, showed the highest antioxidant activity in the protein hydrolysate with IC_{50} value 3.795±0.048 µg/ml. At 0.25 g/ml the highest antioxidant activity of all concentrations of SBP was digested by papain with IC_{50} value 13.807±0.648 µg/ml. Thus, both protein hydrolysates in this condition were selected for further study at a molecular weight cut-off (MWCO). And research of Zhou *et al.* (2012) studied *In vitro* antioxidant activity of enzymatic hydrolysates prepared from abalone *(Haliotis discus hannai Ino)* viscera with five commercially available proteases, including alkali protease, papain, neutral protease, pepsin, and trypsin founded protein hydrolysate prepared by enzyme pepsin have molecule weight more than 200 Da and highest antioxidant activity (IC_{50} value was 5,000 µg/ml).

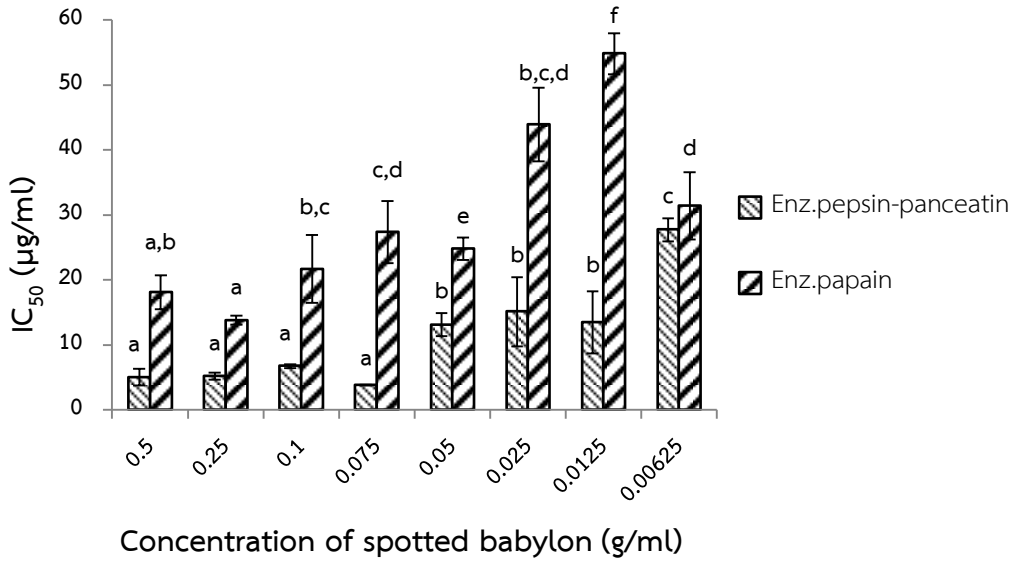


Figure 11 The IC_{50} values of various concentrations of substrate digested by enzymatic hydrolysis of pepsin-pancreatin and papain.

Different values are expressed as mean±SD (n=3) and a, b, c, d, and e are significant at $p > .05$

4.3 Antioxidation activity

4.3.1 DPPH radical scavenging activity

DPPH is a stable free radical used widely in the evaluation of free radical scavenging activity which is known as the easy and quick method. The ability of a reagent to scavenge DPPH radical refers to its antioxidant activity. When a deep purple methanol solution of DPPH receives hydrogen atoms it is converted to a non-radical form, in which the electron on the nitrogen atom is paired and its color changes to pale yellow. This result in the decreasing absorbance observed at 517 nm by the spectrometer reading of the DPPH, due to the delocalization of single unpaired electrons around the whole molecule (Molyneux, 2004). Reasonable free radical scavenging capacities of spotted babylon protein hydrolysate digested by pepsin-pancreatin and papain, as measured by the DPPH assay, were found in a dose-dependent manner (Table 5). However, the 50 % inhibition value (IC_{50} values) for MWCO < 3 kDa showed the lowest IC_{50} value (0.918 ± 0.097 µg/ml digested by pepsin and pancreatin, 1.821±0.035 µg/ml digested by papain), significantly lower than the commonly used positive standard 1 mg/ml of L-ascorbic acid (Vitamin C) (IC_{50} values of 0.0094±0.00012 µg/ml). And research of Sefatie *et al.* (2013) studied *In Vitro* antioxidant activities of protein hydrolysate from Germinated black soybean *(Glycine max L.)* exhibited the highest scavenging activity against 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) free radicals had IC_{50} value was 2,500 µg/mL.

4.3.2 ABTS radical scavenging activity

ABTS assay is tool determining the antioxidant activity of hydrogen donating compounds by scavengers of aqueous phase radical and chain braking antioxidants of the lipid peroxyl radical. The ABTS radical is relatively stable and reduced by antioxidant compounds, most likely hydrogen (Re *et al.*, 1999). The scavenging ability of MWCO < 3 kDa on the ABTS free radical was observed in a dose-dependent

manner (Table 5), but protein hydrolysate digested by pepsin and pancreatin showed a lower activity than the reference standards BHT (butylated hydroxytoluene). Thus, the IC_{50} value of protein hydrolysate digested by pepsin and pancreatin for MWCO < 3 kDa in the ABTS radical scavenging assay was 0.502±0.075 µg/ml, and the IC_{50} value of protein hydrolysate digested by papain for MWCO < 3 kDa was 8.216±0.624a µg/ml, which was higher than BHT (butylated hydroxytoluene). The positive standard used was 500 µg/ml of BHT (butylated hydroxytoluene) (IC_{50} values of 0.142±0.005 µg/ml). And research of Wiriyaphan *et al.* (2012) studied antioxidant activity of protein hydrolysates derived from threadfin bream surimi byproducts showed the highest antioxidant activity based on 2,20-azinobis (3-ethyl-benzothiazoline-6-sulphonate) (ABTS) radical had IC_{50} value was 455 µg/mL.

4.3.3 Nitric oxide scavenging activity

Nitric oxide (NO) is an important chemical mediator generated in biological tissues by specific nitric oxide synthesis which metalizes arginine to citralline with the formation of NO. These compounds are responsible for altering the structural and function behavior of many cellular components. Nitric oxide or radical nitrogen species, formed during their reaction with oxygen, react with nitric oxide thereby inhibiting the generation of nitric (Udenigwe *et al.*, 2009). The best nitric oxide radical scavenging activity was MWCO < 3 kDa of all fractions (IC_{50} 0.420±0.069 µg/ml of protein hydrolysate digested by pepsin and pancreatin, 4.309±0.570 µg/ml of protein hydrolysate digested by papain) (Table 5), which was close to curcumin with an IC_{50} of 0.331±0.006 µg/ml. And research of Rana *et al.* (2010) studied *In vitro* antioxidant and free radical scavenging studies of alcoholic extract of *Medicago sativa l.* estimated by IC_{50} value was : 21.77 µg/mL.

The protein hydrolysate by pepsin-pancreatin was therefore selected for further investigation for purification by RP-HPLC and identification by mass spectrometry.

Table 5 The ultrafiltration separation of spotted babylon protein hydrolysate prepared by pepsin-pancreatin and papain their free radical scavenging capacity assay

Protein hydrolysate digested by Enzyme pepsin-panceatin				Protein hydrolysate digested by Enzyme papain		
Molecular weight cut off	DPPH IC_{50} (µg/ml)	ABTS IC_{50} (µg/ml)	NO IC_{50} (µg/ml)	DPPH IC_{50} (µg/ml)	ABTS IC_{50} (µg/ml)	NO IC_{50} (µg/ml)
Crude protein	14.707 ± 0.508^{c}	21.037 ± 1.472^{e}	5.923 ± 0.141^{b}	136.600 ± 8.903^{e}	422.700 ± 21.717^{d}	110.667 ± 6.137^{e}
Protein hydrolysate	3.795 ± 0.048^{b}	5.650 ± 0.704^{c}	5.422 ± 1.886^{b}	$13.930\pm0.654^{c,d}$	35.693 ± 3.469^{b}	14.913 ± 1.420^{b}
>10kDa	22.98 ± 1.588^{e}	14.143 ± 1.577^{d}	11.203 ± 0.826^{c}	17.670 ± 0.709^{d}	54.483 ± 0.918^{c}	25.707 ± 1.782^{c}
10-5kDa	20.3 ± 1.256^{d}	12.407 ± 2.016^{d}	6.392 ± 0.508^{b}	19.283 ± 1.329^{d}	59.710 ± 1.1497^{c}	34.253 ± 4.868^{d}
<5kDa	3.827 ± 0.352^{b}	2.696 ± 0.465^{b}	1.453 ± 0.211^{a}	$3.889\pm0.306^{a,b}$	15.010 ± 0.674^{a}	8.456 ± 0.511^{a}
5-3 Kda	4.53 ± 0.489^{b}	$2.004\pm0.256^{a,b}$	1.478 ± 0.426^{a}	$8.582\pm0.3561^{b,c}$	33.190 ± 2.581^{b}	21.407 ± 1.184^{c}
<3 Kda	0.918 ± 0.097^{a}	0.502 ± 0.075^{a}	0.420 ± 0.069^{a}	1.821 ± 0.035^{a}	8.216 ± 0.624^{a}	4.309 ± 0.570^{a}
positive control	0.0094 ± 0.00012^{a}	0.142 ± 0.005^{b}	0.331 ± 0.006^{c}	0.0094 ± 0.00012^{a}	0.142 ± 0.005^{b}	0.331 ± 0.006^{c}
	L-ascorbic acid	BHT	Cercumin	L-ascorbic acid	BHT	Cercumin

IC50 values of various concentrations of substrate digested by enzymatic hydrolysis of pepsin-pancreatin and papain. Value are expressed as means±SD (n=3) and a, b, c, d, and e show significant value at p >.05

4.4 Antiproliferation of cancer cell by MTT assays

Results showed that fractions with different molecular masses of spotted babylon protein hydrolysate have different in vitro antioxidant activities (Table 5). Therefore, spotted babylon protein hydrolysate could be a potential source of human antitumor bioactive agents if the results can be repeated *in vivo*. The fraction MWCO < 3 kDa had the highest antioxidant capacity in vitro, and also showed the best antiproliferative activity (Table 6). The conclusion that higher antioxidant activity leads to better antiproliferative activity is consistent with previous reports. These proposed that antioxidants have the potential to prevent and treat diseases associated with active oxygen species, especially some forms of cancers (Jang *et al.*, 2008; Sun *et al.*, 2002). Moreover, the results (Table 5) showed that lung cancer cell line (CHAGO-K1) had the best inhibition activity at MWCO < 3 kDa fraction, with IC_{50} value 0.013±0.003 μg/ml, compared with the positive standard of cancer cell line doxorubicin (IC_{50} value 0.822±0.009 μg/ml). However, results from the study suggest that performing clinical tests on the MWCO < 3 kDa peptide fraction used to test apoptosis cells and identification by RP-HPLC characterization of the specific anticancer peptides, prior to the clinical studies might be a valuable intermediate step. Table 4 IC_{50} values of spotted babylon protein hydrolysates toward five different human malignant cell lines. And research of Kim *et al.* (2012) studied Purification of a novel anticancer peptide from enzymatic hydrolysate of *Mytilus coruscus* showed a novel anticancer peptide The peptide from *M. coruscus* effectively induced cell death on prostate, breast and lung cancer cells but not on normal liver cells (IC_{50} value were 940, 1,410, and 1,220 μg/ml respectively)

Table 6 IC_{50} values of spotted babylon protein hydrolysates toward five different human malignant cell lines.

Size	IC_{50} (µg/ml)				
	BT474	Chago-K1	Hep-G2	KATO-III	SW620
crude protein	235.355±2.90^{d}	754.597±11.923^{b}	823.263±10.341^{d}	679.35±3.918^{e}	671.634±2.534^{g}
Protein hydrolysate	8.753±0.201^{c}	2.486±0.175^{a}	18.128±0.708^{b}	22.296±0.484^{d}	23.742±0.3394^{e}
>10kDa	3.395±0.517^{b}	0.081±0.014^{a}	16.669±0.202^{b}	18.248±0.168^{c}	18.434±0.319^{d}
10-5kDa	6.996±0.775^{c}	8.656±0.273^{a}	32.406±0.227^{c}	22.616±0.222^{d}	28.628±0.196^{f}
<5kDa	1.099±0.152^{a}	1.393±0.036^{a}	3.429±0.195^{a}	2.192±0.167^{a}	3.682±0.217^{b}
5-3 Kda	3.549±0.229^{b}	1.091±0.03^{a}	14.507±0.278^{b}	10.401±0.176^{b}	13.393±0.436^{c}
<3 Kda	0.08±0.016^{a}	0.013±0.003^{a}	0.46367±0.010^{a}	0.379±0.045^{a}	0.553±0.052^{a}
positive control	0.195±0.002^{b}	0.822±0.009^{d}	0.36357±0.009^{c}	1.664±0.006^{e}	0.138±0.003^{a}

IC_{50} values of various concentrations of substrate digested by enzymatic hydrolysis of pepsin- pancreatin. Values are expressed as mean±SD (n=3) and a, b, c, d, and e show significant value at *p >.05*

4.5 Apoptosis of Human lung cancer cell line

Apoptotic cell death is one of the mechanisms by which the rate of cell growth is reduced. This is indicated by cell shrinkage. The results of antiproliferation of cancer cells by MTT assays (section 3.10) showed that the protein hydrolysate of spotted babylon that exhibited the highest antioxidant activity and antiproliferation of cancer cells was the lung cancer cell (CHAGO-K1). Apoptosis was determined by flow cytometer. The lung cancer cell (CHAGO-K1) was incubated with protein hydrolysate at MWCO < 3 kDa (0.0953 µg/ml) and the positive standard doxorubicin (0.00007 µg/ml) for 8 h. Then apoptosis and necrotic cells were measured by Annexin V binding to phosphatidylserine exposed on the external surface of the plasma membrane during the early phase. Propidium iodide (PI) showed late apoptosis with accessible DNA within the nucleus only (Brodie *et al.*, 2003). The results of apoptosis are shown in Figure 12. The control lung cancer cell (CHAGO-K1) untreated with protein hydrolysate (QA) compared to treatment with protein hydrolysate increased necrotic (Q1) and late stage apoptotic cell (Q2). The lung cancer cell (CHAGO-K1) was also treated with the positive standard doxorubicin. The results showed that the lung cancer cell treated with protein hydrolysate and doxorubicin did not increase from the early stage (Q3) of apoptotic cell.

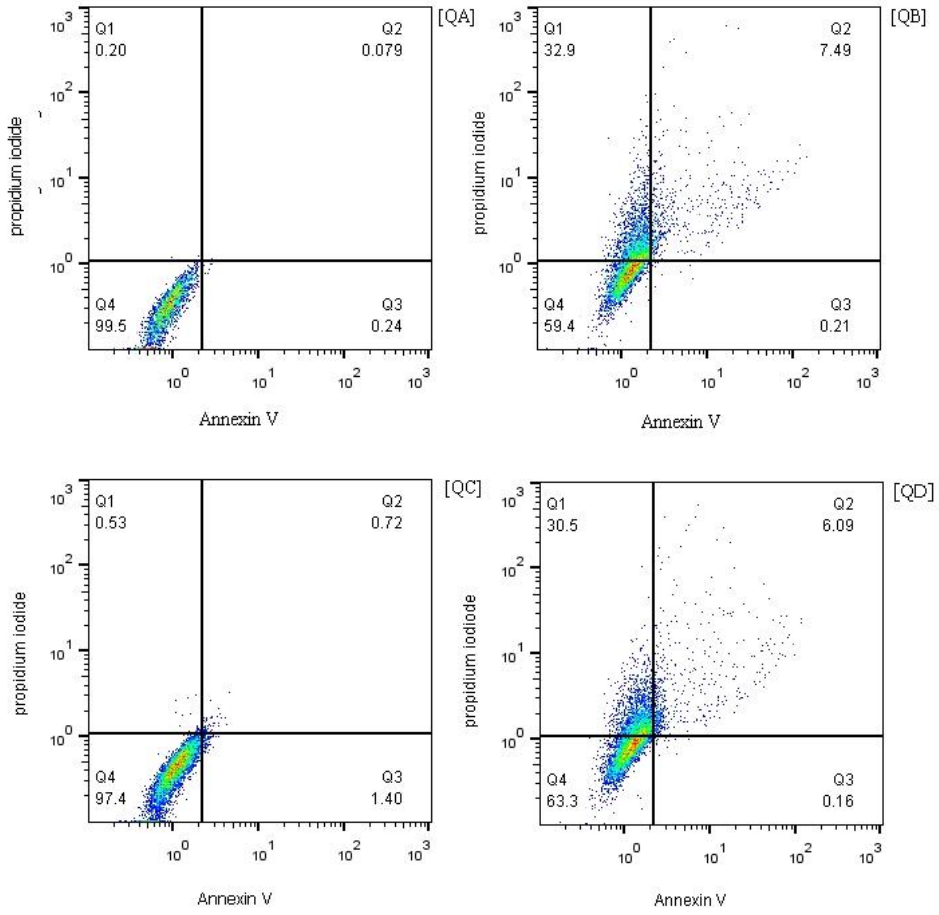


Figure 12 The results of protein hydrolysate from spotted babylon selection determined apoptosis cell by flow cytometer in lung cancer cell.

Q1: Necrotic, Q2: late stage, Q3: early stage and Q4: control stage. QA: untreated lung cancer cell (CHAGO-K1), QB: lung cancer cell (CHAGO-K1) treated with protein hydrolysate, QC: lung cancer cell (CHAGO-K1) treated with doxorubicin without dye, and QD: lung cancer cell (CHAGO-K1) treated with doxorubicin with dye.

4.6 Purification of peptides by RP-HPLC

The MWCO < 3 kDa separated protein hydrolysates of spotted babylon prepared by pepsin-pancreatin were fractionated by reverse-phase high performance liquid chromatography (RP-HPLC) on Luna C_{18} column (4.6 mm × 250 mm) using 0.1 % trifluoroacetic acid (TFA) and acetonitrile (CH_3CN) solvent system. The elution solution was detected at 280 nm. The four fractions of protein hydrolysate of spotted babylon were collected as the chromatographic profile, at retention time 0-10 (fraction A), 10-20 (fraction B), 20-30 (fraction C), and 30-40 (fraction D) (Figure 13). All fractions were incubated and speed vacuumed to remove solvent. The peptide sequence from these fractions was be further characterized by mass spectrometry.

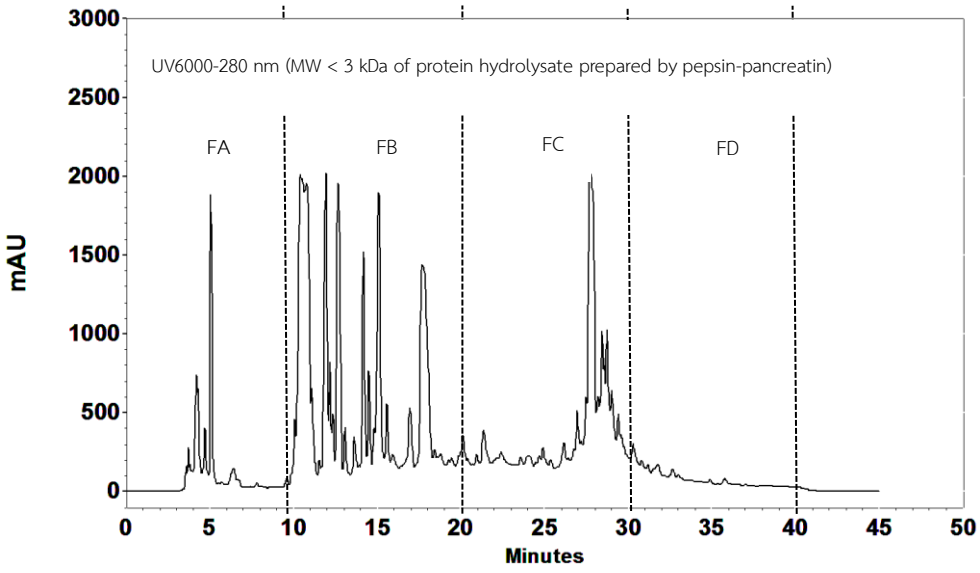


Figure 13 The RP-HPLC chromatogram of MWCO < 3 kDa separated protein hydrolysate from spotted babylon, prepared by pepsin-pancreatin detected at 280 nm.

The fraction liner showed FA: Fraction A (0-10 min), FB: Fraction B (10-20 min), FC: Fraction C (20-30 min) and FD: Fraction D (30-40 min), respectively.

4.7 Identification of antioxidant peptides

The profile of RP-HPLC fraction peptide was isolated from the protein hydrolysate of spotted babylon at MWCO < 3 kDa and the solvent was removed. All fractions were analyzed by the LC-MS/MS method using electrospray ionization (ESI)/ Quadrupole ion trap mass spectrometer to identify peptide antioxidant activity sequences. Results were processed to the MASCOT database. The mass spectrometry results of amino acid sequences are shown in Table 7. The peptide antioxidant activity tested by three methods, DPPH, ABTS, and NO radical scavenging assays, it was tested by DPPH inhibited values of 87% from KIPVYFGK, it was tested by ABTS inhibited values of 33% from GLLIGDDLYAVV and all peptide antioxidant activity was tested by NO radical inhibited values of more 90%.

The antioxidant peptides were processed to the database together with amino acid sequences such as fraction A: KIPVYFGK, B: GLLIGDDLYAVV, C: NRVYVHPF, and EINGKLFLPKYTLSQDVG, and fraction D: WAGGDASGE, MLSNAGVYA, and ETDNGGWTC. The antioxidant peptides at MWCO < 3 kDa of protein hydrolysate from spotted babylon snails prepared by pepsin and pancreatin found amino acid sequences including the hydrophobic group (Leucine (L), Valine (V), Cystine (C), Methionine (M), and Proline (P)), the imidazole functional group (Histidine (H)) and the aromatic group (Tyrosine (Y), Tryptophan (W), and Phenylalanine (F)), with high inhibition antioxidant activity and anticancer cell line. And research of Nazeer *et al.* (2012) *In vitro* and *in vivo* studies on the antioxidant activity of fish peptide isolated from the croaker *(Otolithes ruber)* muscle protein hydrolysate showed purified peptide contained the amino acid sequence as Lys-Thr-Phe-Cys-Gly-Arg-His (861.6 Da), which were expected to contribute to its antioxidant activities with research of Kim *et al.* (2012) was obtained, and the sequence was identified as Ala-Phe-Asn-Ile-His-Asn-Arg-Asn-Leu-Leu. The peptide from *M. coruscus* effectively induced cell death on prostate, breast and lung cancer cells but not on normal liver cells.

Table 7 The results of mass spectrometry of peptide antioxidant activity sequences of protein hydrolysate from spotted babylon prepared pepsin and pancreatin.

Faction *		Accession number	Protein name	Peptide sequence	mass
A	1	2::gi|251274	Rana catesbeiana	KIPVYFGK	965
B	2	2::gi|543379	Rattus norvegicus	GLLIGDDLYAVV	1195
C	3	2::gi|998865	Amphiuma tridactylum	NRVYVHPF	1031
	4	2::gi|226343665	Rhinolophus borneensis	EINGKLFLPKYTLSQDVG	2017
D	5	1::DSIP_RABIT	Oryctolagus cuniculus	WAGGDASGE	848
	6	2::gi|87475034	Salmo salar	MLSNAGVYA	924
	7	2::gi|240018507	Phleocryptes melanops	ETDNGGWTC	989

* fraction retention time was A: 0-10 min, B: 10-20 min, C: 20-30 min, and D: 30-40 min

CHAPTER V
CONCLUSION

The optimal condition of substrate concentration of 0.075 g/ml from hydrolysis showed the highest antioxidant activity by DPPH radical scavenging. This was used as a guideline in this experiment as it has been easily observed, analyzed and widely used before in this kind of study. The results showed that a protein hydrolysate screened substrate concentration at 0.075 g/ml (IC_{50} value 3.795±0.048 µg/ml) exhibited the highest antioxidant activity. After screening the substrate concentration of protein hydrolysate from spotted babylon snails was separated into molecular weight cut-off fractions by ultrafiltration membrane. The seven molecular weight cut-off fractions were tested with various antioxidant activity methods such as DPPH, ABTS, and NO. Radical scavenging activity assays showed high inhibition antioxidant activity at low molecular weight (MWCO < 3 kDa) with IC_{50} values at 0.918±0.097, 0.502±0.075, and 0.420±0.069 µg/ml, respectively. Results showed that protein hydrolysate from spotted babylon prepared by pepsin and pancreatin had lower molecular weight than 3 kDa by donating electrons or hydrogen atoms and reacting with good inhibition free radicals. Protein hydrolysates were tested with antiproliferation and apoptosis of five human malignant cancer cell lines. The results showed that seven fractions of molecular weight cut-off of protein hydrolysate from spotted babylon snails prepared by pepsin and pancreatin can control the growth of different cancer cell lines. The protein hydrolysate at MWCO < 3 kDa which exhibited the best inhibition antiproliferation of cancer cells was the lung cancer cell (CHAGO-K1) with IC_{50} value at 0.013 ± 0.003^{a} µg/ml. The induction of apoptosis was not shown in the early stages but in the late and necrotic stages. The protein hydrolysate of MWCO < 3 kDa fraction was purified by reverse-phase high performance liquid chromatography (RP-HPLC). The peptides antioxidant activities tested by three

methods, DPPH, ABTS, and NO radical scavenging assays, all fractions were highest inhibition antioxidant activity.

REFERENCES

Abdel-Aal, E.-S. (2008). Effects of baking on protein digestibility of organic spelt products determined by two in vitro digestion methods. *Lebensmittel-Wissenschaft & Technologie-Food Science and Technology* 41, 1282-1288.

Ando, T., Mimura, K., Johansson, C.C., Hanson, M.G., Mougiakakos, D., Larsson, C., da Palma, T.M., Sakurai, D., Norell, H., and Li, M. (2008). Transduction with the antioxidant enzyme catalase protects human T cells against oxidative stress. *The Journal of Immunology* 181, 8382-8390.

Battin, E.E., and Brumaghim, J.L. (2009). Antioxidant activity of sulfur and selenium: a review of reactive oxygen species scavenging, glutathione peroxidase, and metal-binding antioxidant mechanisms. *Cell biochemistry and biophysics* 55, 1-23.

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

Bhatnagar, A., Sharma, P.K., and Kumar, N. (2011). A Review on "Imidazoles": Their Chemistry and Pharmacological Potentials. *International Journal of PharmTech Research* 3, 268-282.

Bosch-Morell, F., Roma, J., Marıin, N., Romero, B., Rodriguez-Galietero, A., Johnsen-Soriano, S., Díaz-Llopis, M., and Romero, F.J. (2002). Role of oxygen and nitrogen species in experimental uveitis: anti-inflammatory activity of the synthetic antioxidant ebselen. *Free Radical Biology and Medicine* 33, 669-675.

Botterweck, A., Verhagen, H., Goldbohm, R., Kleinjans, J., and Van Den Brandt, P. (2000). Intake of butylated hydroxyanisole and butylated hydroxytoluene and stomach cancer risk: results from analyses in the Netherlands cohort study. *Food and Chemical Toxicology* 38, 599-605.

Brodie, C., and Blumberg, P.M. (2003). Regulation of cell apoptosis by protein kinase c δ. *Apoptosis* 8, 19-27.

Brown, G., and Borutaite, V. (2006). Interactions between nitric oxide, oxygen, reactive oxygen species and reactive nitrogen species. *Biochemical Society Transactions* 34, 953-956.

Bryan, N.S., and Grisham, M.B. (2007). Methods to detect nitric oxide and its metabolites in biological samples. *Free Radical Biology and Medicine* 43, 645-657.

Chaitanawisuti, N., Kritsanapuntu, S., and Santaweesuk, W. (2011). Effects of dietary protein and lipid levels and protein to energy ratios on growth performance and feed utilization of hatchery-reared juvenile spotted babylon (Babylonia areolata). *Aquaculture international* 19, 13-21.

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.

Chirinang, P., and Intarapichet, K.O. (2009). Amino acids and antioxidant properties of the oyster mushrooms, *Pleurotus ostreatus* and *Pleurotus sajor-caju. Science Asia* 35, 326-331.

Chow, C.K. (1991). Vitamin E and oxidative stress. *Free Radical Biology and Medicine* 11, 215-232.

Cohen, R.A., and Martinez, M.E. (2012). Health Insurance Coverage: Early Release of Estimates From the National Health Interview Survey, January – March 2012.

Dan, S., Shujun, L., Laurie, H.M., Fengmin, Z., Lanfang, Z., Xiaopeng, Z., Wei, L., and Youfu, C. (2010). Effects of high hydrostatic pressure on in vitro digestion of soy protein. *International Agricultural Engineering Journal* 19, 49.

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiological reviews* 82, 47-95.

Edge, R., McGarvey, D.J., and Truscott, T.G. (1997). The carotenoids as anti-oxidants — a review. *Journal of Photochemistry and Photobiology B: Biology* 41, 189-200.

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

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

Hill, A.B. (1965). The environment and disease: association or causation. *Proceedings of the Royal Society of Medicine* 58, 295.

Hitchon, C.A., and El-Gabalawy, H.S. (2004). Oxidation in rheumatoid arthritis. *Arthritis Research and Therapy* 6, 265-278.

Hur, S.J., Lim, B.O., Decker, E.A., and McClements, D.J. (2011). In vitro human digestion models for food applications. *Food Chemistry* 125, 1-12.

Jang, A., Jo, C., Kang, K.S., and Lee, M. (2008). Antimicrobial and human cancer cell cytotoxic effect of synthetic angiotensin-converting enzyme (ACE) inhibitory peptides. *Food Chemistry* 107, 327-336.

Jayan, S., and Rao, M.N.A. (1997). Nitric oxide scavenging by curcuminoids. *Journal of Pharmacy and Pharmacology* 49, 105-107.

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.

Kannan, A., Hettiarachchy, N.S., Marshall, M., Raghavan, S., and Kristinsson, H. (2011). Shrimp shell peptide hydrolysates inhibit human cancer cell proliferation. *Journal of the Science of Food and Agriculture* 91, 1920-1924.

Kashihara, N., Haruna, Y., K Kondeti, V., and S Kanwar, Y. (2010). Oxidative Stress in Diabetic Nephropathy. *Current Medicinal Chemistry* 17, 4256-4269.

Kim, E., Joung, H., Kim, Y., Hwang, J., Ahn, C., Jeon, Y., Moon, S., and Park, P. (2012). Purification of a novel anticancer peptide from enzymatic hydrolysate of *Mytilus coruscus. Journal of microbiology and biotechnology* 22, 1381.

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.

Korhonen, H., and Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal* 16, 945-960.

Kunwar, A., and Priyadarsini, K. (2011). Free radicals, oxidative stress and importance of antioxidants in human health. *J Med Allied Sci* 1, 53-60.

Lakshmi, S., Padmaja, G., Kuppusamy, P., and Kutala, V. (2009). Oxidative stress in cardiovascular disease. *Indian journal of biochemistry & biophysics* 46, 421-440.

Lambert, C.R., Black, H.S., and Truscott, T.G. (1996). Reactivity of butylated hydroxytoluene. *Free Radical Biology and Medicine* 21, 395-400.

Lee, H.S., Park, C.B., Kim, J.M., Jang, S.A., Park, I.Y., Kim, M.S., Cho, J.H., and Kim, S.C. (2008). Mechanism of anticancer activity of buforin IIb, a histone H2A-derived peptide. *Cancer letters* 271, 47-55.

Maher, S., and McClean, S. (2006). Investigation of the cytotoxicity of eukaryotic and prokaryotic antimicrobial peptides in intestinal epithelial cells in vitro. *Biochemical Pharmacology* 71, 1289-1298.

Mamboya, E.A.F. (2012). Papain, a plant enzyme of biological importance: A review. *American Journal of Biochemistry and Biotechnology* 8, 99-104.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in plant science* 7, 405-410.

Mohamed, T.K., Issoufou, A., and Zhou, H. (2012). Antioxidant activity of fractionated foxtail millet protein hydrolysate. *International Food Research Journal* 19, 207-213.

Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin J Sci Technol* 26, 211-219.

Mora-Escobedo, R., del Carmen Robles-Ramírez, M., Ramón-Gallegos, E., and Reza-Alemán, R. (2009). Effect of protein hydrolysates from germinated soybean on cancerous cells of the human cervix: an in vitro study. *Plant foods for human nutrition* 64, 271-278.

Moreno, C.S. (2002). Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Science and Technology International* 8, 121-137.

Mundi, S., and Aluko, R.E. (2014). Inhibitory Properties of Kidney Bean Protein Hydrolysate and its Membrane Fractions Against Renin, Angiotensin Converting

Enzyme, and Free Radicals. *Austin Journal of Nutrition and Food Sciences* 2, 1-11.

Murray, J.C., Symonds, P., Ward, W., Huggins, M., Tiga, A., Rice, K., Heng, Y.M., Todd, I., and Robins, R.A. (2004). Colorectal cancer cells induce lymphocyte apoptosis by an endothelial monocyte-activating polypeptide-II-dependent mechanism. *The Journal of Immunology* 172, 274-281.

Nazeer, R.A., Sampath Kumar, N.S., and Jai Ganesh, R. (2012). In vitro and in vivo studies on the antioxidant activity of fish peptide isolated from the croaker *(Otolithes ruber)* muscle protein hydrolysate. *Peptides* 35, 261-268.

Neurath, H., and Walsh, K.A. (1976). Role of proteolytic enzymes in biological regulation (a review). *Proceedings of the National Academy of Sciences* 73, 3825-3832.

Oberley, L.W., and Buettner, G.R. (1979). Role of superoxide dismutase in cancer: a review. *Cancer research* 39, 1141-1149.

Ozben, T. (2007). Oxidative stress and apoptosis: impact on cancer therapy. *Journal of pharmaceutical sciences* 96, 2181-2196.

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.

Pham-Huy, L.A., He, H., and Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. *International journal of biomedical science: IJBS* 4, 89.

Rahman, I. (2005). Oxidative stress in pathogenesis of chronic obstructive pulmonary disease. *Cell biochemistry and biophysics* 43, 167-188.

Rana, M., Katbamna, R., Padhya, A., Dudhrejiya, A., Jivani, N., and Sheth, N. (2010). In vitro antioxidant and free radical scavenging studies of alcoholic extract of *Medicago sativa l. Rom J Biol plant biol* 55, 15-22.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine* 26, 1231-1237.

Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., Cui, C., Kakuda, Y., and Xue, S.J. (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.

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

Seafood Health Facts. (2008). Making Smart Choices Balancing the Benefits and Risks of Seafood Consumption. *Resources for Health Care Providers and Patients*:january1, http://seafoodhealthfacts.org/seafood_nutrition/practitioners/composition_chart.php.

Sefatie, R.S., Fatoumata, T., Eric, K., Shi, Y.H., and Guo-wei, L. (2013). In vitro antioxidant activities of protein hydrolysate from germinated black soybean *(Glycine max L.). Food Science and Technology* 5, 453-459.

Shai, Y. (1999). Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1462, 55-70.

Sies, H. (1997). Oxidative stress: oxidants and antioxidants. *Experimental physiology* 82, 291-295.

Silvestre, M.P.C. (1997). Review of methods for the analysis of protein hydrolysates. *Food Chemistry* 60, 263-271.

Sisein, E.A. (2014). Biochemistry of free fadicals and antioxidants. *Sch Acad J Biosci* 2, 110-118.

Stadtman, E.R., and Levine, R.L. (2003). Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino acids* 25, 207-218.

Su, L., Xu, G., Shen, J., Tuo, Y., Zhang, X., Jia, S., Chen, Z., and Su, X. (2010). Anticancer bioactive peptide suppresses human gastric cancer growth through modulation of apoptosis and the cell cycle. *Oncology reports* 23, 3-9.

Sun, J., Chu, Y.F., Wu, X., and Liu, R.H. (2002). Antioxidant and antiproliferative activities of common fruits. *Journal of Agricultural and Food Chemistry* 50, 7449-7454.

Suprenant, K.A., and Rebhun, L.I. (1984). Purification and characterization of oocyte cytoplasmic tubulin and meiotic spindle tubulin of the surf clam *Spisula solidissima. The Journal of cell biology* 98, 253-266.

Udenigwe, C.C., Lu, Y.L., Han, C.H., Hou, W.C., and Aluko, R.E. (2009). Flaxseed protein-derived peptide fractions: Antioxidant properties and inhibition of lipopolysaccharide-induced nitric oxide production in murine macrophages. *Food Chemistry* 116, 277-284.

Uttara, B., Singh, A., Zamboni, P., and Mahajan, R. (2009). Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Current neuropharmacology* 7, 65-74.

Wang, B., Li, Z.-R., Chi, C.-F., Zhang, Q.-H., and Luo, H.-Y. (2012). Preparation and evaluation of antioxidant peptides from ethanol-soluble proteins hydrolysate of Sphyrna lewini muscle. *Peptides* 36, 240-250.

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, G., Li, X., and Wang, Z. (2009). APD2: the updated antimicrobial peptide database and its application in peptide design. *Nucleic acids research* 37, D933-D937.

Wiriyaphan, C., Chitsomboon, B., and Yongsawadigul, J. (2012). Antioxidant activity of protein hydrolysates derived from threadfin bream surimi by products. *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.

Yang, B., Yang, H., Li, J., Li, Z., and Jiang, Y. (2011). Amino acid composition, molecular weight distribution and antioxidant activity of protein hydrolysates of soy sauce lees. *Food Chemistry* 124, 551-555.

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.

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 discus hannai Ino)* viscera. *Food and Bioproducts Processing* 90, 148-154.

Zhou, D.Y., Tang, Y., Zhu, B.W., Qin, L., Li, D.M., Yang, J.F., Lei, K., and Murata, Y. (2012). Antioxidant activity of hydrolysates obtained from scallop *(Patinopecten yessoensis)* and abalone *(Haliotis discus hannai Ino)* muscle. *Food Chemistry* 132, 815-822.

APPENDIX

APPENDIX A

Bradford assay

1. Bradford stock solution

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

2. Bradford working buffer

Distilled water	425 ml
95 %Ethanol	15 ml
88% phosphoric	30 ml
Bradford stock solution	30 ml

3. Bradford stock solution filtered through filter paper (Whatman No.1 paper) at store at room temperature and in brown glass bottle. Usable for several weeks, but may need to be re-filtered.

APPENDIX B

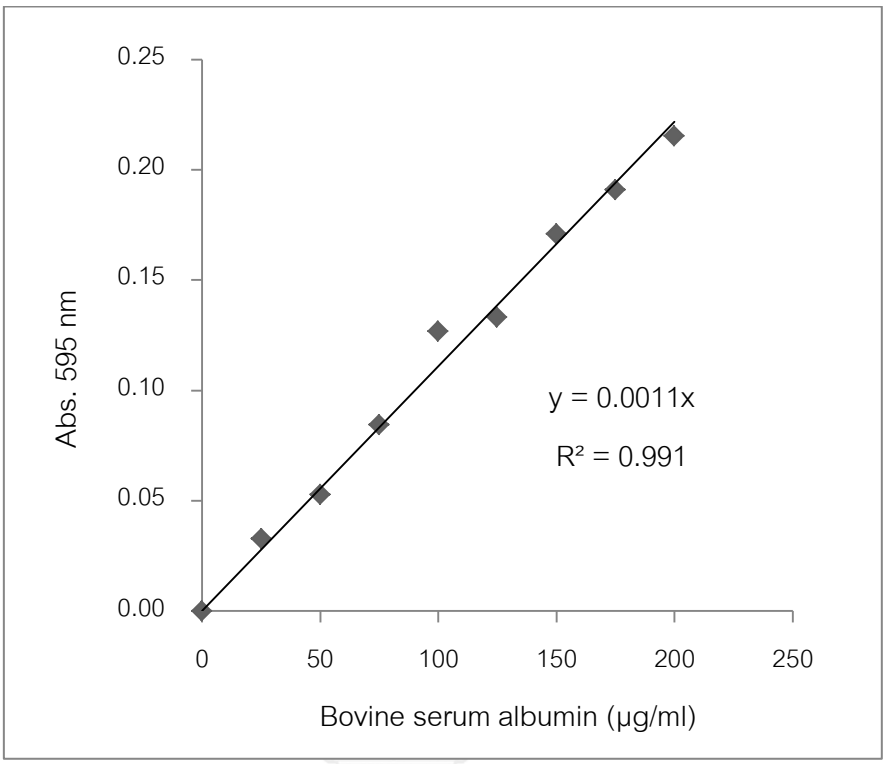


Figure 14 Standard Curve protein of Bovine serum albumin (BSA 1mg/ml) determinate by Bradford method.

APPENDIX C

-20 mM potassium phosphate buffer (pH 7.2)

20 mM Potassium dihydrogen phosphate (KH_2PO_4)	2.72 g/l
20 mM Dipotassium phosphate (K_2HPO_4)	3.49 g/l
0.15 M Sodium chloride (NaCl)	8.77 g/l
0.00015 Sodium azide (NaN_3)	0.01 g/l

-Adjusted pH 7.2 with 1 M hydrogen chloride (HCl) and 1 M sodium hydroxide (NaOH), stirred until dissolved and adjusted volume to 1 L with distilled water (DI water).

APPENDIX D

Peptide View

-Fraction A (1) (0-10 min)

MS/MS Fragmentation of **KIPVYFGK**

Found in **gi|251274** in NCBInr, S-modulin [Rana catesbeiana]

Monoisotopic mass of neutral peptide Mr(calc): 950.5589

Match to Query 164: 950.485448 from (476.250000, 2+) intensity (6801118.0000) index (173)

Title: Cmpd 174, +MSn (476.3), 9.6 min

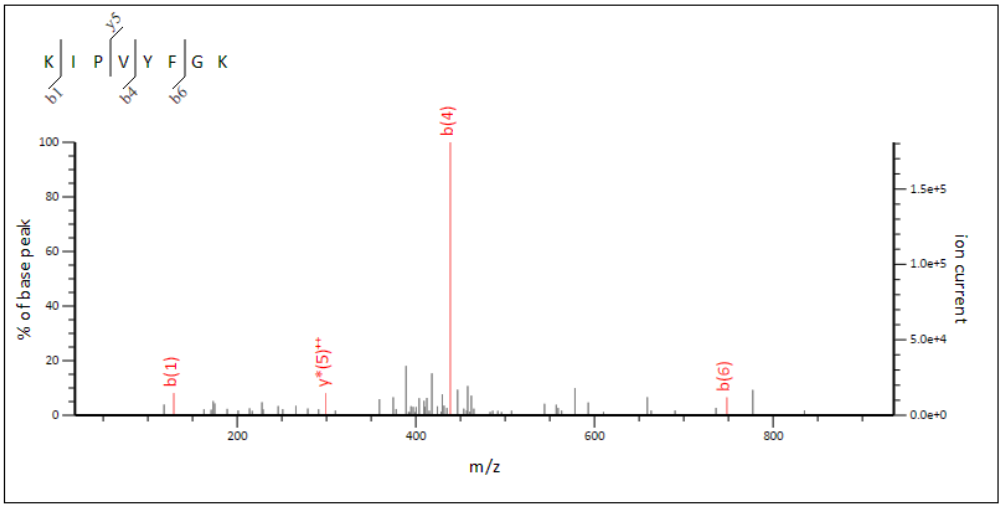


Figure 15 Fraction A (1) (0-10 min)

-Fraction B (2) (10-20 min)

MS/MS Fragmentation of **GLLIGDDLYAVV**

Found in **gi|543379** in NCBInr, cytochrome P450 UT-7b - rat (fragment)

Monoisotopic mass of neutral peptide Mr(calc): 1246.6809

Match to Query 21: 1245.788172 from (416.270000,3+) intensity(195031.0000) index(90)

Title: Cmpd 91, +MSn(416.3), 2.1 min

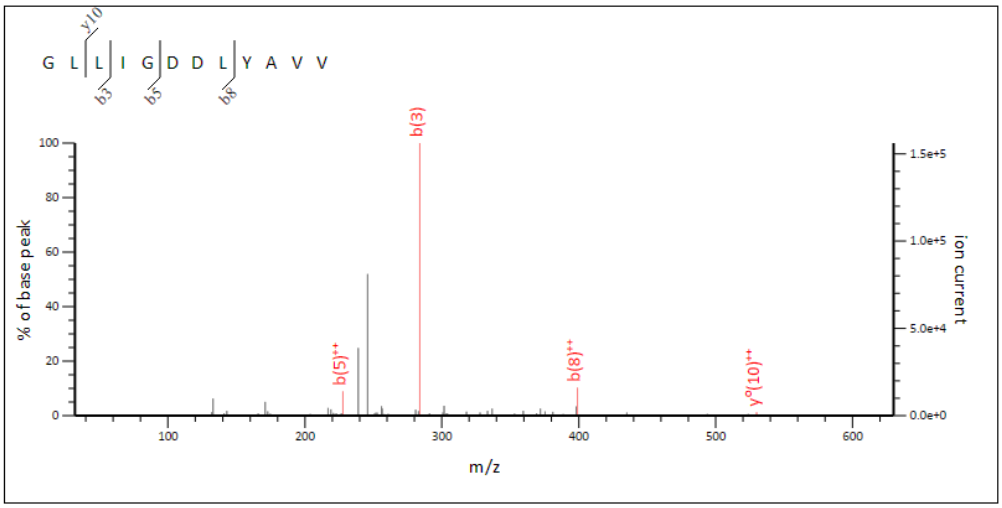


Figure 16 Fraction B (2) (10-20 min)

-Fraction C (3) (20-30 min)

MS/MS Fragmentation of **NRVYVHPF**

Found in **gi|998865** in NCBInr, [Asn1,Val5]angiotensin II [Amphiuma tridactylum=three-toed amphiumas, plasma, Peptide, 8 aa]

Monoisotopic mass of neutral peptide Mr(calc): 1030.5349

Match to Query 160: 1030.505448 from (516.260000,2+) intensity(13280392.0000) index(166)

Title: Cmpd 167, +MSn(516.3), 9.1 min

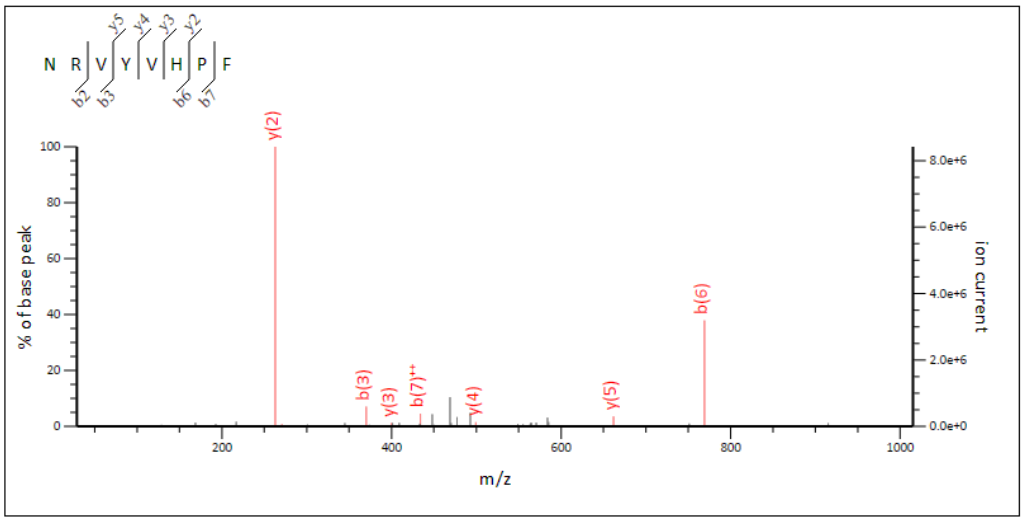


Figure 17Fraction C (3) (20-30 min)

-Fraction C (4) (20-30 min)

MS/MS Fragmentation of **EINGKLFLPKYTLSQDVG**

Found in **gi|226343665** in NCBInr, thyrotropin, partial [Rhinolophus borneensis]

Monoisotopic mass of neutral peptide Mr(calc): 2021.0833

Match to Query 69: 2019.908172 from (674.310000,3+) intensity(1499970.0000) index(130)

Title: Cmpd 131, +MSn(674.3), 5.1 min

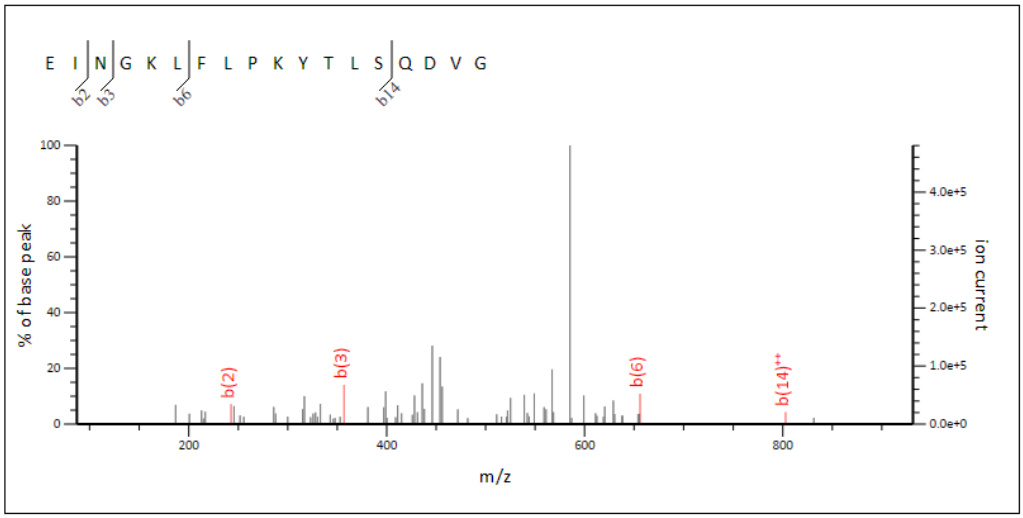


Figure 18 Fraction C (4) (20-30 min)

-Fraction D (5) (30-40 min)

MS/MS Fragmentation of **WAGGDASGE**

Found in **DSIP_RABIT** in SwissProt, Delta sleep-inducing peptide OS=Oryctolagus cuniculus PE=1 SV=1

Monoisotopic mass of neutral peptide Mr(calc): 848.3301

Match to Query 97: 848.425448 from (425.220000,2+) intensity(829358.0000) index(95)

Title: Cmpd 96, +MSn(425.2), 13.9 min

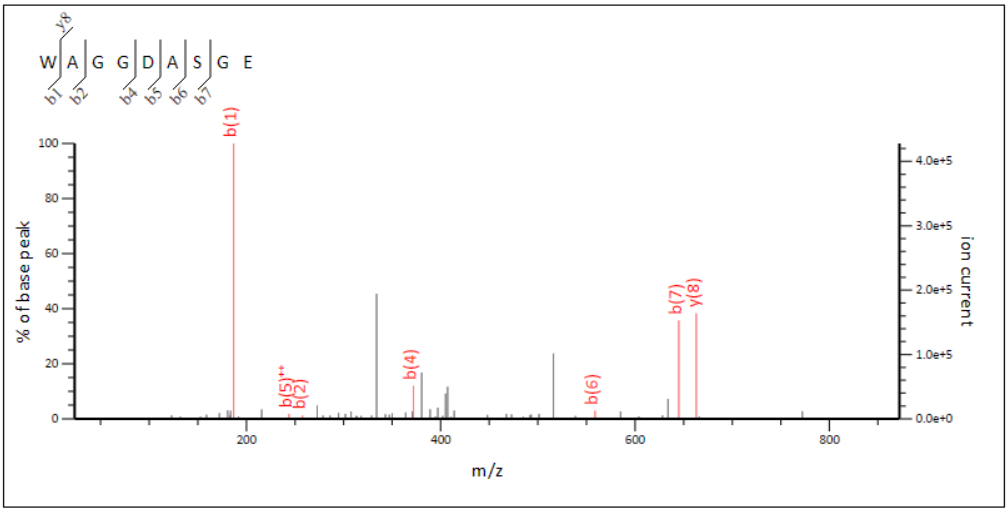


Figure 19 Fraction D (5) (30-40 min)

-Fraction D (6) (30-40 min)

MS/MS Fragmentation of **MLSNAGVYA**

Found in **gi|87475034** in NCBInr, aryl hydrocarbon receptor 2 beta [Salmo salar]

Monoisotopic mass of neutral peptide Mr(calc): 924.4375

Match to Query 117: 925.445448 from (463.730000,2+) intensity(1371209.0000) index(94)

Title: Cmpd 95, +MSn(463.7), 13.9 min

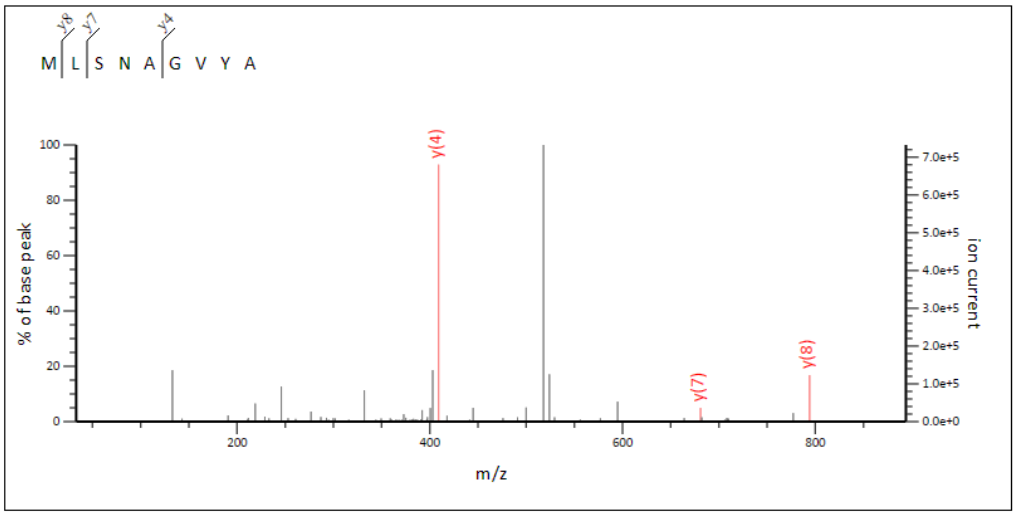


Figure 20 Fraction D (6) (30-40 min)

-Fraction D (7) (30-40 min)

MS/MS Fragmentation of **ETDNGGWTC**

Found in **gi|240018507** in NCBInr, beta-fibrinogen, partial [Phleocryptes melanops]

Monoisotopic mass of neutral peptide Mr(calc): 981.3498

Match to Query 124: 981.098172 from (328.040000,3+) intensity(1178560.0000) index(72)

Title: Cmpd 73, +MSn(328.0), 13.2 min

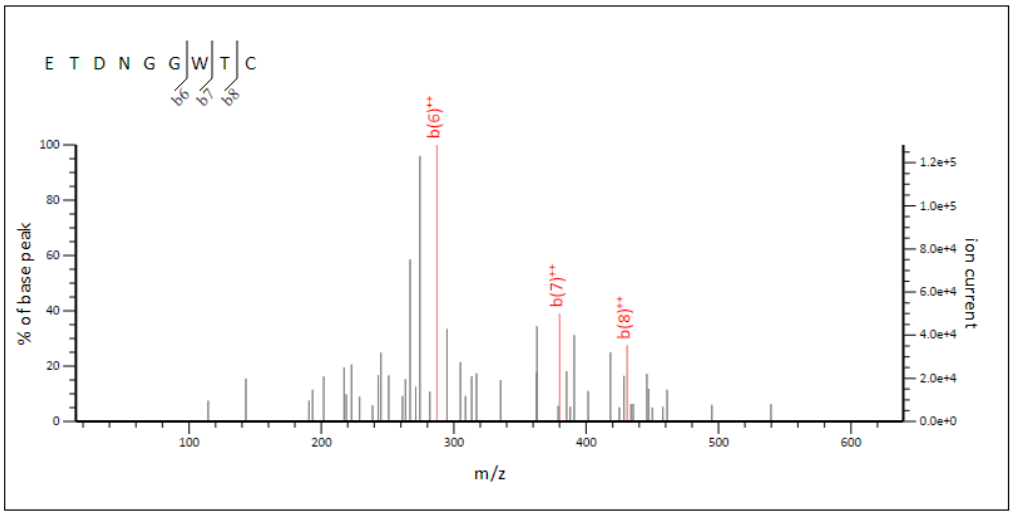


Figure 21 Fraction D (7) (30-40 min)

VITA

Miss Putcha Petsantad was born on October 10, 1989 in Rayong, Thailand. She received Bachelor Degree of Science in 20011 from Department of Biotechnology, Faculty of Marine Technology, Burapha University, Thailand. She has further studied to the Master Degree of Science in Biotechnology, the Faculty of Science, Chulalongkorn University in 2011.

Academic presentation

1. Petsantat, P., Chaitanawisuti, N., and Karnchanatat, A.* Antioxidation and antiproliferative activities of protein hydrolysate from spotted babylon (Babylonia areolata). In “7th Asia Oceania Human Proteome Organization (AOHUPO) Congress and the 9th International Symposium of the Protein Society of Thailand” 6-8 August 2014, Miracle Grand Convention Hotel, Bangkok, THAILAND (Proceeding book).